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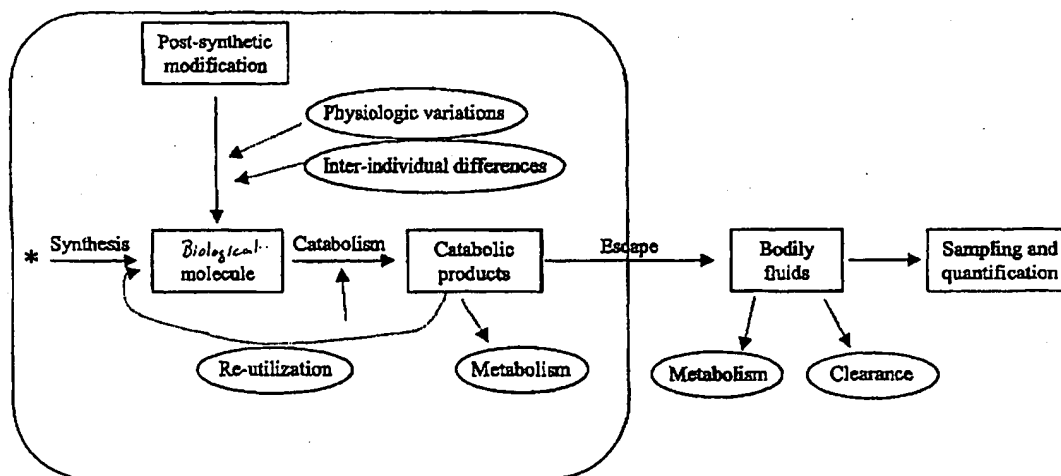
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(54) Title: MEASUREMENT OF BIOSYNTHESIS AND BREAKDOWN RATES OF BIOLOGICAL MOLECULES THAT ARE INACCESSIBLE OR NOT EASILY ACCESSIBLE TO DIRECT SAMPLING, NON-INVASIVELY, BY LABEL INCORPORATION INTO METABOLIC DERIVATIVES AND CATABOLITIC PRODUCTS



(57) Abstract: Methods of determining rate of biosynthesis or breakdown of biological molecules from metabolic derivatives and catabolic products are disclosed herein. In particular, methods of measuring the rates of biosynthesis and breakdown of biological molecules inaccessible or not easily accessible to direct sampling by sampling metabolic derivatives and catabolic products in accessible biological samples are disclosed herein.

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**MEASUREMENT OF BIOSYNTHESIS AND BREAKDOWN RATES OF  
BIOLOGICAL MOLECULES THAT ARE INACCESSIBLE OR NOT  
EASILY ACCESSIBLE TO DIRECT SAMPLING, NON-INVASIVELY, BY  
LABEL INCORPORATION INTO METABOLIC DERIVATIVES AND  
CATABOLITIC PRODUCTS**

**CROSS-REFERENCE TO RELATED APPLICATIONS**

This application claims priority to 60/356,008 filed on February 12, 2002.

**FIELD OF THE INVENTION**

This invention relates techniques for the measurement of the rate of biosynthesis and breakdown of biological molecules and polymers. More particularly, it relates to techniques for measuring the rates of biosynthesis and breakdown of biological molecules, especially those of the polymeric class and in tissues or other locations that are inaccessible or not easily accessible to direct sampling, in a non-invasive manner in individuals without having to sample the biological molecules directly in the tissues of interest.

**BACKGROUND OF THE INVENTION**

Publications referred to by reference numbering in this specification correspond to the reference list at the end of the specification.

Various ways of measuring rates of synthesis or breakdown of biological polymers and other biological molecules of interest have been described. One such invasive method involves the collection of tissues by various invasive procedures, *e.g.*, surgical excision, percutaneous biopsy, post-mortem analysis, or other sampling procedures (termed "invasive" procedures herein), after administration of an isotopically labeled precursor molecule, then isolation of the polymer or other molecule of interest from the tissue so collected, followed by measurement of the

isotopic content or labeling pattern in said polymer or other molecule and calculation of the synthesis or breakdown rate of said polymer or other molecule based on the rate of isotope incorporation. This method has some disadvantages that include, *inter alia*, the need for invasive tissue measurements with attendant medical risk, discomfort, need for expert medical involvement, and limitations on the number of measurements that can be performed.

Another invasive method involves repeated collection of tissue by the invasive procedures listed above after an intervention with measurement of the content (concentration or pool size) of the polymer or other molecule of interest in each tissue sample, and calculation of the rate of change in the net pool size over time, thereby determining the net synthesis (accrual) or net breakdown (depletion) rate. This method has some disadvantages that include, *inter alia*, the need for repeated measurements and the lack of a true synthetic or breakdown rate measured, with instead a net accrual or depletion rate generated. Further, it is a well-recognized principle in biochemistry that net changes in concentration (accrual or depletion) are not identical to and do not reveal true or absolute rates of synthesis or breakdown (7), because concurrent synthesis and breakdown (herein termed "turnover") is not measured or accounted for by net changes in concentration.

A method that is commonly used in medical practice involves the indirect estimate of pool size or concentration, and their changes over time, by use of repeated radiographic measurements (*e.g.*, x-rays or dual-energy-X-ray absorptiometry for estimating bone mass (4); nuclear magnetic resonance imaging or computerized tomography for estimating muscle or fat mass (9); radiographic procedures for estimating tumor mass). This approach suffers from the same limitations as direct biochemical measurements of concentrations or pool sizes of molecules (noted above), in addition to limitations of accuracy.

Another non-invasive method that has been used involves the collection of a breakdown product that is specific for and derived from a biological molecule or

other molecule of interest and that is secreted or excreted into blood or urine, and calculation of the breakdown rate of the biological molecule or other molecule based on the recovery of said breakdown product. (10, 11). This method has some disadvantages that include, *inter alia*, the inability to measure synthesis rates or true breakdown rates, rather than a net release rate, and other technical limitations that are well-described for these methods (e.g., incomplete recovery of breakdown products due to their biological clearance and catabolism in the organism; interference by delayed or unpredictable excretion of the breakdown products; etc).

The disadvantages and limitations of these prior methods for measuring the synthesis and breakdown rates of biological molecules located in inaccessible tissues inaccessible biological samples are substantial and have held back important fields, including medical diagnostics, drug discovery, genetics, functional genomics and basic research. The disadvantages noted here are not intended to be comprehensive; many other limitations and disadvantages of these methods exist and could be mentioned.

An optimal non-invasive method of measuring rates of biosynthesis and breakdown rates of biological molecules would have the following characteristics: accuracy, capacity to measure true or absolute rates of biosynthesis or breakdown (*i.e.*, accounts for turnover), and does not require total quantitative collection of breakdown products (*i.e.* metabolic derivatives and catabolic products). Furthermore, an ideal method would allow constant isotope levels in the precursor pool to be maintained for prolonged periods of time in a simple, non-demanding manner, for example, on the order of a few half-lives of long-lived molecules. However, there has not been a technique that has fulfilled these objectives. A method for measuring non-invasively the rates of synthesis or breakdown of biological molecules that are inaccessible or not easily accessible to direct sampling (e.g. molecules in or associated with tissues of the internal organs) and that is widely applicable, reliable, easy to perform, inexpensive, without toxicities or complications, applicable in

human subjects, free of the need for medical supervision or in-patient procedures (such as intravenous infusions), does not require complex instructions, and possesses the advantages of simple interpretation, therefore would be extremely valuable and useful in fields ranging from medical diagnostics to drug discovery, genetics, functional genomics, and basic research.

### BRIEF SUMMARY OF THE INVENTION

In order to meet these needs, the present invention is directed to a method of determining the rates of biosynthesis and breakdown of biological molecules that are inaccessible or not easily accessible to direct sampling, such as intracellular or extracellular molecules in the tissues of internal organs, in a non-invasive manner.

In one aspect, the present invention is directed to a method for determining the rate of biosynthesis or breakdown of one or more biological molecules in an individual comprising the steps of: administering an isotope-labeled precursor molecule to an individual for a period of time sufficient for the label of the isotope-labeled precursor molecule to become incorporated into the one or more biological molecules; obtaining one or more biological samples from an individual, wherein the one or more biological samples comprise one or more metabolic derivatives of said one or more biological molecules as resulting from *in vivo* metabolism of the biological molecules; and detecting the incorporation of the label in said one or more metabolic derivatives by mass spectrometry to determine said rate of biosynthesis or breakdown of the one or more biological molecules.

In another aspect, the present invention is directed to a method for determining the rate of biosynthesis or breakdown of one or more biological molecules in an individual comprising the steps of: administering an isotope-labeled precursor molecule to an individual for a period of time sufficient for the label of the isotope-labeled precursor molecule to become incorporated into the one or more inaccessible biological molecules; obtaining one or more accessible biological samples from an

individual, wherein the one or more accessible biological samples comprise one or more metabolic derivatives of said one or more inaccessible biological molecules as resulting from *in vivo* metabolism of the inaccessible biological molecules; and detecting the incorporation of the label in said one or more metabolic derivatives by mass spectrometry to determine said rate of biosynthesis or breakdown of the one or more inaccessible biological molecules.

The detecting step may include calculating the isotope enrichment of the one or more inaccessible biological molecules by mass isotopomer distribution analysis (MIDA) and applying precursor-product or exponential decay equations to determine the rate of biosynthesis or breakdown of the inaccessible biological molecule.

The precursor molecules may be administered *in vivo*. In another variation, the isotopic label is selected from the group including  $^2\text{H}$ ,  $^3\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^{18}\text{O}$ ,  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{35}\text{S}$ ,  $^{32}\text{P}$ ,  $^{125}\text{I}$ , and  $^{131}\text{I}$ . In a further variation, the label is  $^2\text{H}$ .

The precursor molecule may be water.

The method may include the additional step of partially purifying the one or more metabolic derivatives from the biological samples.

The isotope-labeled precursor molecule may be administered orally.

The method may include the additional step of degrading the one or more metabolic derivatives to form degraded metabolic derivatives. In further variation, the degraded metabolic derivatives are further separated by gas chromatography or HPLC.

The individual may be a human.

The metabolic derivatives may be catabolic products. In a further variation, the metabolic derivative derives primarily from the one or more biological molecules.

The label of the isotope-labeled precursor molecule is incorporated into the one or more metabolic derivatives biosynthetic incorporation into the one or more biological molecules followed by catabolic breakdown of the one or more biological molecules to form the one or more metabolic derivatives.

In another variation, the one or more metabolic derivatives cannot be utilized in the biosynthesis of another biological molecule in the individual.

In a further variation, biosynthesis or breakdown does not occur in the one or more biological samples.

The method may include the additional step of discontinuing the administering step.

The one or more biological molecules may be selected from the group including proteins, polynucleotides, lipids, glycosaminoglycans, prostoglycans, and carbohydrates.

The one or more biological molecules may be proteins. In further variation, the precursor molecule is an amino acid or one or more metabolic precursors of an amino acid. In further variation, label is incorporated post-translationally into the protein. In a still further variation, one or more metabolic derivatives is an amino acid or peptide.

The biological molecule may be collagen. In further variation, one or more metabolic derivatives may include one or more of the following collagen-specific metabolic derivatives: pyridinoline, deoxypyridinoline, hydroxyproline, hydroxylysine, glucosylgalactosyl-hydroxylysine, galactosylhydroxylysine, N-terminal telopeptide  $\alpha$ (I) (SEQ ID NO:1), N-terminal telopeptide  $\alpha$ 2(I) (SEQ ID NO:2), N-terminal telopeptide  $\alpha$ 2(I) (SEQ ID NO:3), N-terminal telopeptide  $\alpha$ 1(II) (SEQ ID NO:4), N-terminal telopeptide  $\alpha$ 1(III) (SEQ ID NO:5), C-terminal telopeptide  $\alpha$ 1(I) (SEQ ID NO:6), C-terminal telopeptide  $\alpha$ 2(I) (SEQ ID NO:7), C-terminal telopeptide  $\alpha$ 1(II) (SEQ ID NO:8), C-terminal telopeptide  $\alpha$ 1(II) (SEQ ID NO:9), C-terminal telopeptide  $\alpha$ 1(II) (SEQ ID NO:10), C-terminal telopeptide  $\alpha$ 1(III) (SEQ ID NO:11), cross-linked carboxy-terminal peptide of type I collagen (ICTP), PINP( $\alpha$ 1) (SEQ ID NO:12), PICP( $\alpha$ 1) (SEQ ID NO:13), PINP( $\alpha$ 2) (SEQ ID NO:14), PICP( $\alpha$ 2) (SEQ ID NO:15), PIINP( $\alpha$ 1) (SEQ ID NO:16), PIICP( $\alpha$ 1) (SEQ ID NO:17), PIINP( $\alpha$ 1) (SEQ ID NO:18), PIICP( $\alpha$ 1)(SEQ ID NO:19),

PIVNP( $\alpha$ 1)(SEQ ID NO:20), PIVNP( $\alpha$ 2)(SEQ ID NO:21), PIVNP( $\alpha$ 2)(SEQ ID NO:22), PIVNP( $\alpha$ 3) (SEQ ID NO:23), PIVNP( $\alpha$ 4) (SEQ ID NO:24), PIVNP( $\alpha$ 5) (SEQ ID NO:25), and PIVNP( $\alpha$ 6) (SEQ ID NO:26). In a still further variation, the one or more metabolic derivatives are an N-terminal or C-terminal amino acid sequence specific to a type of collagen.

The biological molecule may be myosin. In a further variation, the metabolic derivative is 3-methylhistidine.

The protein may be Amyloid Precursor Protein (APP). In a further variation, the metabolic derivative may be an APP-specific metabolic derivative. In a further variation, the APP-specific metabolic derivative is amyloid-beta 1-40 (SEQ ID NO:27) or amyloid-beta 1-42 (SEQ ID NO:28), or APP C peptide.

The one or more metabolic derivatives may be a post-translationally modified amino acid or protein.

The metabolic derivative may be one or more of a phosphorylated, methylated, hydroxylated, glycosylated, N-acetyl-glucosaminated, prenylated, palmitoylated, and gamma-carboxylated amino acids or peptides.

The biological molecule may be myelin basic protein. In further variation, the protein is brain myelin basic protein. In a still further variation, the metabolic derivative is myelin basic protein-like material.

The biological sample may be urine.

The one or more biological molecules may be a lipid. In another variation, the lipid is a brain membrane lipid. In a still further variation, the metabolic derivative is -24(s)-hydroxycholesterol.

The one or more biological molecules may be a polynucleotide. In a further variation, the polynucleotide is deoxyribonucleic acid (DNA). In a further variation, the label is introduced post-replication to the DNA. In an additional variation, the one or more metabolic derivatives is a nucleic acid with one or more nucleic acid residues. In yet another variation, the metabolic derivative is selected from the group



including methyl-cytosine, a methylated base, 8-oxo-guanosine, an oxidatively modified base, deoxyribose, and ribose.

The one or more biological molecules may be a glycosaminoglycan or proteoglycan. In a further variation, one or more metabolic derivatives is one or more of hyaluronic acid disaccharide, hyaluronic acid polymers, N-acetyl glucosamine, N-acetyl-galactosamine, chondroitin-sulfate disaccharide, chondroitin-sulfate polymers, heparin sulfate disaccharide, and heparin sulfate disaccharide polymers.

The biological sample may be an accessible biological sample.

The precursor molecule may be administered repeatedly or continuously over a defined period of time.

The invention may be directed to a method of identifying a disease state by assessing the biosynthesis or breakdown rate, wherein the rate is indicative of a disease state. In another variation, the disease state is a physiological condition characterized by an alteration in the biosynthesis or breakdown rate of the one or more biological molecules. In another variation, the disease state or condition is one or more of osteoporosis, left-ventricular hypertrophy, liver cirrhosis, liver fibrosis, congestive heart failure, scleroderma, black-lung (coal-miner's pneumoconiosis), cardiac fibrosis, lung fibrosis, Alzheimer's disease, multiple sclerosis, rheumatoid arthritis, diabetes mellitus, muscle wasting syndromes, muscular dystrophies, athletic training, and cancer.

In another variation, the invention is directed to a method for monitoring a response of a disease state or a condition in an individual to a therapeutic intervention by assessing the rate of biosynthesis or breakdown, before the initiation of the therapeutic intervention; assessing the rate of biosynthesis or breakdown of the one or more biological molecules after the initiation of such therapeutic intervention; and comparing the rates of synthesis or breakdown before and after therapeutic intervention to monitor the response of a disease or a condition to therapeutic intervention. In another variation, the disease state or condition is one or more of

osteoporosis, left-ventricular hypertrophy, liver cirrhosis, liver fibrosis, congestive heart failure, scleroderma, black-lung (coal-miner's pneumoconiosis), cardiac fibrosis, lung fibrosis, Alzheimer's disease, multiple sclerosis, rheumatoid arthritis, diabetes mellitus, muscle wasting syndromes, muscular dystrophies, athletic training, and cancer.

In a further variation, the invention may be directed to a method for determining a whole-body pool size of the one or more biological molecules in an individual by measuring the rate of biosynthesis of the biological molecule; measuring the biosynthesis rate of the one or more biological molecules; and dividing the daily excretion rate by the daily fractional replacement rate of the one or more metabolic derivatives to calculate whole-body pool size of the one or more biological molecules in the individual.

The invention also may be directed to a kit for determining the biosynthetic rate or breakdown rate of one or more biological molecules in an individual including an isotope-labeled precursor, and instructions for use of the kit, wherein the kit is used to determine the rate of biosynthesis or breakdown of the one or more biological molecules in the individual. In another variation, the kit may include chemical compounds for isolating the one or more metabolic derivatives from urine, bone, or muscle. In further variation, the kit may include a tool for administration of precursor molecules. The kit may also include an instrument for collecting a sample from the subject.

#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a flow chart of an embodiment of the invention.

Figure 2 summarizes schematically the advantages of the current invention over previous non-isotopic uses of post-synthetically modified metabolic derivatives to estimate turnover of tissue molecules. The circled items with broken-line arrows represent factors that could alter reliability or interpretability of previous methods.

Figure 3 shows the rise-to-plateau principle. In Figure 3A, label (\*) enters pool A (precursor pool) and pool B (product) is synthesized from A. The replacement rate constant ( $k$ ) for pool B is revealed by the shape of the rise-to-plateau curve, as shown here for  $k = 0.1, 0.5$  and  $1.0 \text{ d}^{-1}$ . The plateau value of labeling reached in pool B will depend upon the fraction of B derived from the precursor pool. Examples of 50% (left) and 100% B (right) deriving from endogenous synthesis are shown.

Figure 4 depicts enrichments of  $^2\text{H}_2\text{O}$  in body water of representative human subjects who drank 50-100 ml of  $^2\text{H}_2\text{O}$  daily for 10-12 weeks. The data show that the precursor pool of body water is stable over a period of weeks for each subject.

Figure 5 depicts a time course of body  $^2\text{H}_2\text{O}$  enrichments in rats maintained on 4% drinking water after baseline priming bolus to 2.5-3.0% body water enrichment.

Figure 6A-B depict pathways of labeled hydrogen exchange from labeled water into selected free amino acids. Two nonessential amino acids (alanine, glycine) and an essential amino acid (leucine) are shown, by way of example. Alanine and glycine are presented in Figure 6A. Leucine is presented in Figure 6B. Abbreviations: TA, transaminase; PEP-CK, phosphoenol-pyruvate carbokinase; TCAC, tricarboxylic acid cycle; STHM, serine tetrahydrofolate methyl transferase. Figure 6C depicts  $\text{H}_2^{18}\text{O}$  labeling of free amino acids for protein synthesis.

Figure 7 depicts a schematic model for measurement of new protein synthesis from the incorporation of hydrogen-labeled  $\text{H}_2\text{O}$  (\*H) into protein-bound amino acids. Labeled hydrogens are represented by closed circles; unlabeled by open circles. The expected time course of labeling each compartment (body water, free amino acids, protein-bound amino acids) is shown in the inset.

Figure 8A depicts the course of  $^2\text{H}$  incorporation from  $^2\text{H}_2\text{O}$  into galactose moiety of brain galactosyl cerebroside in mice maintained on 8%  $^2\text{H}_2\text{O}$  as drinking water. Each time point represents five mice. EM1 is the excess abundance of M+1 mass isotopomer in methyl tetraacetyl galactose. Figure 8B depicts a comparison of  $^2\text{H}$

incorporation into galactose moiety of galactosyl-cerebroside in brain versus blood from a mouse maintained on 8%  $^2\text{H}_2\text{O}$  in drinking water.

Figure 9 depicts a gas chromatograph/mass spectrum of methyl, triacetyl acetate, N-acetyl glucosamine derivative of hyaluronic acid (HA).

## DETAILED DESCRIPTION OF THE INVENTION

Applicants have discovered an effective method for determining the rates of biosynthesis and/or breakdown of biological molecules in a non-invasive manner. In particular, applicants have discovered a method of determining the rates of biosynthesis and breakdown of biological molecules that are inaccessible or not easily accessible to direct sampling, such as intracellular or extracellular molecules in the tissues of internal organs, in a non-invasive manner.

### I. General Techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, *Molecular Cloning: A Laboratory Manual*, second edition (Sambrook et al., 1989) Cold Spring Harbor Press; *Oligonucleotide Synthesis* (M.J. Gait, ed., 1984); *Methods in Molecular Biology*, Humana Press; *Cell Biology: A Laboratory Notebook* (J.E. Cellis, ed., 1998) Academic Press; *Animal Cell Culture* (R.I. Freshney, ed., 1987); *Introduction to Cell and Tissue Culture* (J.P. Mather and P.E. Roberts, 1998) Plenum Press; *Cell and Tissue Culture: Laboratory Procedures* (A. Doyle, J.B. Griffiths, and D.G. Newell, eds., 1993-8) J. Wiley and Sons; *Methods in Enzymology* (Academic Press, Inc.); *Handbook of Experimental Immunology* (D.M. Weir and C.C. Blackwell, eds.); *Gene Transfer Vectors for Mammalian Cells* (J.M. Miller and M.P. Calos, eds., 1987); *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987);

*PCR: The Polymerase Chain Reaction*, (Mullis et al., eds., 1994); *Current Protocols in Immunology* (J.E. Coligan et al., eds., 1991); *Short Protocols in Molecular Biology* (Wiley and Sons, 1999); and *Mass isotopomer distribution analysis at eight years: theoretical, analytic and experimental considerations* by Hellerstein and Neese (*Am J Physiol* 276 (*Endocrinol Metab.* 39) E1146-E1162, 1999). Furthermore, procedures employing commercially available assay kits and reagents will typically be used according to manufacturer-defined protocols unless otherwise noted.

## II. Definitions

Unless otherwise defined, all terms of art, notations and other scientific terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this invention pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the art. The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, *Mass isotopomer distribution analysis at eight years: theoretical, analytic and experimental considerations* by Hellerstein and Neese (*Am J Physiol* 276 (*Endocrinol Metab.* 39) E1146-E1162, 1999). As appropriate, procedures involving the use of commercially available kits and reagents are generally carried out in accordance with manufacturer defined protocols and/or parameters unless otherwise noted.

“Isotopomers” refer to isotopic isomers or species that have identical elemental compositions but are constitutionally and/or stereochemically isomeric because of isotopic substitution, for example  $\text{CH}_3\text{NH}_2$ ,  $\text{CH}_3\text{NHD}$  and  $\text{CH}_2\text{DNH}_2$ .

“Isotopologues” refer to isotopic homologues or molecular species that have identical elemental and chemical compositions but differ in isotopic content (e.g.,  $\text{CH}_3\text{NH}_2$  vs.  $\text{CH}_3\text{NHD}$  in the example above). Isotopologues are defined by their

isotopic composition, therefore each isotopologue has a unique exact mass but may not have a unique structure. An isotopologue is usually comprised of a family of isotopic isomers (isotopomers) which differ by the location of the isotopes on the molecule (*e.g.*,  $\text{CH}_3\text{NHD}$  and  $\text{CH}_2\text{DNH}_2$  are the same isotopologue but are different isotopomers).

"Mass isotopomer" refers to a family of isotopic isomers that are grouped on the basis of nominal mass rather than isotopic composition. A mass isotopomer may include molecules of different isotopic compositions, unlike an isotopologue (*e.g.*,  $\text{CH}_3\text{NHD}$ ,  $^{13}\text{CH}_3\text{NH}_2$ ,  $\text{CH}_3^{15}\text{NH}_2$  are part of the same mass isotopomer but are different isotopologues). In operational terms, a mass isotopomer is a family of isotopologues that are not resolved by a mass spectrometer. For quadrupole mass spectrometers, this typically means that mass isotopomers are families of isotopologues that share a nominal mass. Thus, the isotopologues  $\text{CH}_3\text{NH}_2$  and  $\text{CH}_3\text{NHD}$  differ in nominal mass and are distinguished as being different mass isotopomers, but the isotopologues  $\text{CH}_3\text{NHD}$ ,  $\text{CH}_2\text{DNH}_2$ ,  $^{13}\text{CH}_3\text{NH}_2$ , and  $\text{CH}_3^{15}\text{NH}_2$  are all of the same nominal mass and hence are the same mass isotopomers. Each mass isotopomer is therefore typically composed of more than one isotopologue and has more than one exact mass. The distinction between isotopologues and mass isotopomers is useful in practice because all individual isotopologues are not resolved using quadrupole mass spectrometers and may not be resolved even using mass spectrometers that produce higher mass resolution, so that calculations from mass spectrometric data must be performed on the abundances of mass isotopomers rather than isotopologues. The mass isotopomer lowest in mass is represented as  $M_0$ ; for most organic molecules, this is the species containing all  $^{12}\text{C}$ ,  $^1\text{H}$ ,  $^{16}\text{O}$ ,  $^{14}\text{N}$ , etc. Other mass isotopomers are distinguished by their mass differences from  $M_0$  ( $M_1$ ,  $M_2$ , etc.). For a given mass isotopomer, the location or position of isotopes within the molecule is not specified and may vary (*i.e.*, "positional isotopomers" are not distinguished).

“Mass isotopomer pattern” refers to a histogram of the abundances of the mass isotopomers of a molecule. Traditionally, the pattern is presented as percent relative abundances where all of the abundances are normalized to that of the most abundant mass isotopomer; the most abundant isotopomer is said to be 100%. The preferred form for applications involving probability analysis, however, is proportion or fractional abundance, where the fraction that each species contributes to the total abundance is used (see below). The term isotope pattern is sometimes used in place of mass isotopomer pattern, although technically the former term applies only to the abundance pattern of isotopes in an element.

A “monomer” refers to a chemical unit that combines during the synthesis of a polymer and which is present two or more times in the polymer.

A “polymer” refers to a molecule synthesized from and containing two or more repeats of a monomer.

A “peptide” is a sequence of two or more amino acids.

A “metabolic derivative” refers to any molecule produced from the biochemical conversion of one molecule into a related molecule by a reaction or series of reactions. A metabolic derivative in this context includes, but is not limited to, catabolic products as defined herein. It is understood that a metabolic derivative in this context includes, but is not limited to, any metabolic product deriving from a biological molecule including, but not limited to, metabolic products derived from amino acids, proteins, nucleic acids, lipids, carbohydrates, glycosaminoglycans, proteoglycans, porphyrins, and functional and non-functional fragments thereof.

A “catabolic product” refers to any compound that is produced as a result of catabolism. Catabolism generally refers to a process in which a molecule is broken into smaller parts, as opposed to anabolism in which molecules are synthesized from smaller subunits. Catabolic products are a subset of metabolic derivatives.

A “post-synthetic catabolite” (or “post-synthetic catabolic product”) refers to a molecule or compound that has undergone the following biochemical sequence: 1)

biosynthetic incorporation into an biological molecule; 2) catabolic breakdown of said biological molecule; 3) release from said biological molecule into an accessible biological sample or a biological fluid.

An "individual" is a vertebrate, preferably a mammal, more preferably a human.

A "isotope-labeled precursor molecule" refers to any molecule that contains an isotope of an element at levels above that found in natural abundance molecules.

As used herein, an individual "at risk" is an individual who is considered more likely to develop a disease state or a physiological state than an individual who is not at risk. An individual "at risk" may or may not have detectable symptoms indicative of the disease or physiological condition, and may or may not have displayed detectable disease prior to the treatment methods (*e.g.*, therapeutic intervention) described herein. "At risk" denotes that an individual has one or more so-called risk factors. An individual having one or more of these risk factors has a higher probability of developing one or more disease(s) or physiological condition(s) than an individual without these risk factor(s). These risk factors can include, but are not limited to, history of family members developing one or more diseases, related conditions, or pathologies, history of previous disease, age, sex, race, diet, presence of precursor disease, genetic (*i.e.*, hereditary) considerations, and environmental exposure.

"Labeled Water" includes water labeled with one or more specific heavy isotopes of either hydrogen or oxygen. Specific examples of labeled water include  $^2\text{H}_2\text{O}$ ,  $^3\text{H}_2\text{O}$ , and  $\text{H}_2^{18}\text{O}$ .

"Partially purifying" refers to methods of removing one or more components of a mixture of other similar compounds. For example, "partially purifying a protein or peptide" refers to removing one or more proteins or peptides from a mixture of one or more proteins or peptides.

"Isolating" refers to separating one compound from a mixture of compounds. For example, "isolating a protein or peptide" refers to separating one specific protein or



peptide from all other proteins or peptides in a mixture of one or more proteins or peptides.

An "accessible biological sample" encompasses a variety of sample types obtained from an individual through minimally invasive or non-invasive approaches (e.g., urine collection, blood drawing, needle aspiration, and other procedures involving minimal risk, discomfort or effort). The definition also includes samples that have been manipulated in any way after their procurement (through minimally invasive or non-invasive approaches), such as by treatment with reagents, solubilization, or enrichment for certain components, such as proteins or polynucleotides. The term "accessible biological sample" includes, but is not limited to, urine, blood, saliva, lacrimal fluid, inflammatory exudates, synovial fluid, abscess, empyema or other infected fluid, cerebrospinal fluid, sweat, pulmonary secretions (sputum), seminal fluid, and feces.

"Inaccessible biological sample" refers to biological samples that are not easily obtained through minimally invasive or non-invasive approaches. Inaccessible biological samples may be collected by invasive procedures, such as surgical excision, percutaneous biopsies, and post-mortem analysis. In particular, inaccessible biological samples include the internal organs (such as liver, heart, kidney, lung, pancreas, intestine, spleen, brain, bone marrow, skeletal muscle), the intracellular space of tissues of the internal organs, and the extracellular matrix of internal organs (such as bone, cartilage, joint space, ground substance, basement membrane, and vessel wall).

An "inaccessible biological molecule" refers to a biological molecule that cannot be easily acquired from or detectable in an accessible biological samples.

### III. Methods of the Invention

The invention includes a method that allows measurement of biosynthesis and breakdown rates of a wide variety of biological molecules, including polymers of

various classes, including, but not limited to proteins, lipids, carbohydrates, nucleic acids, glycosaminoglycans, and proteoglycans, which are important in biology and disease.

In one aspect, the invention includes a method for determining the rate of biosynthesis or breakdown of the biological molecule in an individual by detecting the incorporation of isotope label in metabolic derivatives of the biological molecule. The biological molecule may be any molecule including, but not limited to, proteins and peptides, polynucleotides (such as DNA and RNA), lipids (such as cholesterol), carbohydrates, glycosaminoglycans, proteoglycans, combinations or polymers thereof, or biological molecules in other chemical classes.

Preferably, the biological molecules are inaccessible biological molecules and/or are from inaccessible biological samples. The rate of biosynthesis or breakdown of the inaccessible biological molecules may be measured by measuring metabolic derivatives or catabolic products.

Moreover, the metabolic derivatives or catabolic products of the biological molecules are preferably in an accessible biological sample. The metabolic derivatives or catabolic products also preferably derive primarily, and optionally uniquely, from the biological molecule. Thus, the metabolic derivatives or catabolic products preferably identify or characterize, the biological molecule. Preferably, only small quantities, and not the total quantity, of metabolic derivatives or catabolic products need to be acquired. Further, the metabolic derivatives or catabolic products preferably cannot be re-incorporated into other biological molecules via metabolism.

*A. Administering to an Individual an Isotope-Labeled Precursor Molecule*

*1. Labeled precursor molecules*

*a. Isotope labels*

As illustrated in Figure 1, the first step in measuring biosynthesis, breakdown, and/or turnover rates involve administering an isotope-labeled precursor molecule to an individual. The isotope labeled precursor molecule may be a stable isotope or

radioisotope. Isotope labels that can be used include, but are not limited to,  $^2\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^{18}\text{O}$ ,  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{35}\text{S}$ ,  $^{32}\text{P}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ , or other isotopes of elements present in organic systems.

In one embodiment, the isotope label is  $^2\text{H}$ .

*b. Precursor Molecules*

The precursor molecule may be any molecule that is metabolized in the body to form a biological molecule. Isotope labels may be used to modify all precursor molecules disclosed herein to form isotope-labeled precursor molecules.

The entire precursor molecule may be incorporated into one or more biological molecules. Alternatively, a portion of the precursor molecule may be incorporated into one or more biological molecules.

Precursor molecules may include, but not limited to,  $\text{CO}_2$ ,  $\text{NH}_3$ , glucose, lactate,  $\text{H}_2\text{O}$ , acetate, fatty acids.

*i. Water as a Precursor Molecule*

Water is a precursor of proteins, polynucleotides, lipids, carbohydrates, modifications or combinations thereof, and other biological molecules. As such, labeled water may serve as a precursor in the methods taught herein.

Labeled water may be readily obtained commercially. For example,  $^2\text{H}_2\text{O}$  may be purchased from Cambridge Isotope Labs (Andover, MA), and  $^3\text{H}_2\text{O}$  may be purchased, e.g., from New England Nuclear, Inc. In general,  $^2\text{H}_2\text{O}$  is non-radioactive and thus, presents fewer toxicity concerns than radioactive  $^3\text{H}_2\text{O}$ .  $^2\text{H}_2\text{O}$  may be administered, for example, as a percent of total body water, e.g., 1% of total body water consumed (e.g., for 3 litres water consumed per day, 30 microliters  $^2\text{H}_2\text{O}$  is consumed). If  $^3\text{H}_2\text{O}$  is utilized, then a non-toxic amount, which is readily determined by those of skill in the art, is administered.

Relatively high body water enrichments of  $^2\text{H}_2\text{O}$  (e.g., 1-10% of the total body water is labeled) may be achieved using the techniques of the invention. This water enrichment is relatively constant and stable as these levels are maintained for

weeks or months in humans and in experimental animals without any evidence of toxicity (Figures 3-5). This finding in a large number of human subjects (> 100 people) is contrary to previous concerns about vestibular toxicities at high doses of  $^2\text{H}_2\text{O}$ . Applicants have discovered that as long as rapid changes in body water enrichment are prevented (e.g., by initial administration in small, divided doses), high body water enrichments of  $^2\text{H}_2\text{O}$  can be maintained with no toxicities. For example, the low expense of commercially available  $^2\text{H}_2\text{O}$  allows long-term maintenance of enrichments in the 1-5% range at relatively low expense (e.g., calculations reveal a lower cost for 2 months labeling at 2%  $^2\text{H}_2\text{O}$  enrichment, and thus 7-8% enrichment in the alanine precursor pool (Figures 6A-B), than for 12 hours labeling of  $^2\text{H}$ -leucine at 10% free leucine enrichment, and thus 7-8% enrichment in leucine precursor pool for that period).

Relatively high and relatively constant body water enrichments for administration of  $\text{H}_2^{18}\text{O}$  may also be accomplished, since the  $^{18}\text{O}$  isotope is not toxic, and does not present a significant health risk as a result (Figure 6C).

Labeled water may be used as a near-universal precursor for most classes of biological molecules.

#### ii. *Protein, Oligonucleotide, Lipid, and Carbohydrate Precursors*

In another embodiment, precursor molecules are precursors of proteins, polynucleotides, lipids, and carbohydrates.

##### *Precursors of Proteins*

The precursor molecule may be any protein precursor molecule known in the art. These precursor molecules may be  $\text{CO}_2$ ,  $\text{NH}_3$ , glucose, lactate,  $\text{H}_2\text{O}$ , acetate, and fatty acids.

Precursor molecules of proteins may also include one or more amino acids. The precursor may be any amino acid. The precursor molecule may be a singly or multiply deuterated amino acid. The precursor molecule is one or more of  $^{13}\text{C}$ -lysine,  $^{15}\text{N}$ -histidine,  $^{13}\text{C}$ -serine,  $^{13}\text{C}$ -glycine,  $^2\text{H}$ -leucine,  $^{15}\text{N}$ -glycine,  $^{13}\text{C}$ -leucine,  $^2\text{H}_5$ -

histidine, and any deuterated amino acid. Labeled amino acids may be administered, for example, undiluted with non-deuterated amino acids. All isotope labeled precursors may be purchased commercially, for example, from Cambridge Isotope Labs (Andover, MA).

The precursor molecule may also include any precursor for post-translational or pre-translationally modified amino acids. These precursors include but are not limited to precursors of methylation such as glycine, serine or  $\text{H}_2\text{O}$ ; precursors of hydroxylation, such as  $\text{H}_2\text{O}$  or  $\text{O}_2$ ; precursors of phosphorylation, such as phosphate,  $\text{H}_2\text{O}$  or  $\text{O}_2$ ; precursors of prenylation, such as fatty acids, acetate,  $\text{H}_2\text{O}$ , ethanol, ketone bodies, glucose, or fructose; precursors of carboxylation, such as  $\text{CO}_2$ ,  $\text{O}_2$ ,  $\text{H}_2\text{O}$ , or glucose; precursors of acetylation, such as acetate, ethanol, glucose, fructose, lactate, alanine,  $\text{H}_2\text{O}$ ,  $\text{CO}_2$ , or  $\text{O}_2$ ; and other post-translational modifications known in the art.

The degree of labeling present in free amino acids may be determined experimentally, or may be assumed based on the number of labeling sites in an amino acid. For example, when using hydrogen isotopes as a label, the labeling present in C-H bonds of free amino acid or, more specifically, in tRNA-amino acids, during exposure to  $^2\text{H}_2\text{O}$  in body water may be identified. The total number of C-H bonds in each non essential amino acid is known - e.g. 4 in alanine, 2 in glycine, etc.

The precursor molecule for proteins may be water. The hydrogen atoms on C-H bonds are the hydrogen atoms on amino acids that are useful for measuring protein synthesis from  $^2\text{H}_2\text{O}$  since the O-H and N-H bonds of peptides and proteins are labile in aqueous solution. As such, the exchange of  $^2\text{H}$ -label from  $^2\text{H}_2\text{O}$  into O-H or N-H bonds occurs without the synthesis of proteins from free amino acids as described above. C-H bonds undergo incorporation from  $\text{H}_2\text{O}$  into free amino acids during specific enzyme-catalyzed intermediary metabolic reactions (Figure 6). The presence of  $^2\text{H}$ -label in C-H bonds of protein-bound amino acids after  $^2\text{H}_2\text{O}$  administration therefore means that the protein was assembled from amino acids that were in the free

form during the period of  $^2\text{H}_2\text{O}$  exposure - i.e. that the protein is newly synthesized. Analytically, the amino acid derivative used must contain all the C-H bonds but must remove all potentially contaminating N-H and O-H bonds.

Hydrogen atoms from body water may be incorporated into free amino acids.  $^2\text{H}$  or  $^3\text{H}$  from labeled water can enter into free amino acids in the cell through the reactions of intermediary metabolism, but  $^2\text{H}$  or  $^3\text{H}$  cannot enter into amino acids that are present in peptide bonds or that are bound to transfer RNA. Free essential amino acids may incorporate a single hydrogen atom from body water into the  $\alpha$ -carbon C-H bond, through rapidly reversible transamination reactions (Figure 6). Free non-essential amino acids contain a larger number of metabolically exchangeable C-H bonds, of course, and are therefore expected to exhibit higher isotopic enrichment values per molecule from  $^2\text{H}_2\text{O}$  in newly synthesized proteins (Figures 6A-B).

One of skill in the art will recognize that labeled hydrogen atoms from body water may be incorporated into other amino acids via other biochemical pathways. For example, it is known in the art that hydrogen atoms from water may be incorporated into glutamate via synthesis of the precursor  $\alpha$ -ketoglutarate in the citric acid cycle. Glutamate, in turn, is known to be the biochemical precursor for glutamine, proline, and arginine. By way of another example, hydrogen atoms from body water may be incorporated into post-translationally modified amino acids, such as the methyl group in 3-methyl-histidine, the hydroxyl group in hydroxyproline or hydroxylysine, and others. Other amino acids synthesis pathways are known to those of skill in the art.

Oxygen atoms ( $\text{H}_2^{18}\text{O}$ ) may also be incorporated into amino acids through enzyme-catalyzed reactions. For example, oxygen exchange into the carboxylic acid moiety of amino acids may occur during enzyme catalyzed reactions. Incorporation of labeled oxygen into amino acids is known to one of skill in the art as illustrated in Figure 6C. Oxygen atoms may also be incorporated into amino acids from  $^{18}\text{O}_2$  through enzyme catalyzed reactions (including hydroxyproline, hydroxylysine or other post-translationally modified amino acids).

Hydrogen and oxygen labels from labeled water may also be incorporated into amino acids through post-translational modifications. In one embodiment, the post-translational modification may already include labeled hydrogen or oxygen through biosynthetic pathways prior to post-translational modification. In another embodiment, the post-translational modification may incorporate labeled hydrogen, oxygen, carbon, or nitrogen from metabolic derivatives involved in the free exchange labeled hydrogens from body water, either before or after post-translational modification step (e.g. methylation, hydroxylation, phosphorylation, prenylation, sulfation, carboxylation, acetylation or other known post-translational modifications).

#### *Precursors of Polynucleotides*

The precursor molecule may include components of polynucleotides. Polynucleotides include purine and pyrimidine bases and a ribose-phosphate backbone. The precursor molecule may be any polynucleotide precursor molecule known in the art.

The precursor molecules of polynucleotides may be  $\text{CO}_2$ ,  $\text{NH}_3$ , urea,  $\text{O}_2$ , glucose, lactate,  $\text{H}_2\text{O}$ , acetate, ketone bodies and fatty acids, glycine, succinate or other amino acids, and phosphate.

Precursor molecules of polynucleotides may also include one or more nucleoside residues. The precursor molecules may also be one or more components of nucleoside residues. Glycine, aspartate, glutamine, and tetrahydrofolate, for example, may be used as precursor molecules of purine rings. Carbamyl phosphate and aspartate, for example, may be used as precursor molecules of pyrimidine rings. Adenine, adenosine, guanine, guanosine, cytidine, cytosine, thymine, or thymidine may be given as precursor molecules for deoxyribonucleosides. All isotope labeled precursors may be purchased commercially, for example, from Cambridge Isotope Labs (Andover, MA).

The precursor molecule of polynucleotides may be water. The hydrogen atoms on C-H bonds of polynucleotides, polynucleosides, and nucleotide or nucleoside

precursors may be used to measure polynucleotide synthesis from  $^2\text{H}_2\text{O}$ . C-H bonds undergo exchange from  $\text{H}_2\text{O}$  into polynucleotide precursors. The presence of  $^2\text{H}$ -label in C-H bonds of polynucleotides, nucleosides, and nucleotide or nucleoside precursors, after  $^2\text{H}_2\text{O}$  administration therefore means that the polynucleotide was synthesized during this period. The degree of labeling present may be determined experimentally, or assumed based on the number of labeling sites in a polynucleotide or nucleoside.

Hydrogen atoms from body water may be incorporated into free nucleosides or polynucleotides.  $^2\text{H}$  or  $^3\text{H}$  from labeled water can enter these molecules through the reactions of intermediary metabolism.

One of skill in the art will recognize that labeled hydrogen atoms from body water may be incorporated into other polynucleotides, nucleotides, or nucleosides via various biochemical pathways. For example, glycine, aspartate, glutamine, and tetrahydrofolate, which are known precursors molecules of purine rings. Carbamyl phosphate and aspartate, for example, are known precursor molecules of pyrimidine rings. Ribose and ribose phosphate, and their synthesis pathways, are known precursors of polynucleotide synthesis.

Oxygen atoms ( $\text{H}_2^{18}\text{O}$ ) may also be incorporated into polynucleotides, nucleotides, or nucleosides through enzyme-catalyzed biochemical reactions, including those listed above. Oxygen atoms from  $^{18}\text{O}_2$  may also be incorporated into nucleotides by oxidative reactions, including non-enzymatic oxidation reactions (including oxidative damage, such as formation of 8-oxo-guanine and other oxidized bases or nucleotides).

Isotope-labeled precursors may also be incorporated into polynucleotides, nucleotides, or nucleosides in post-replication modifications. Post-replication modifications include modifications that occur after synthesis of DNA molecules. The metabolic derivatives may be methylated bases, including, but not limited to, methylated cytosine. The metabolic derivatives may also be oxidatively modified



bases, including, but not limited to, 8-oxo-guanosine. Those of skill in the art will readily appreciate that the label may be incorporated during synthesis of the modification.

#### *Precursors of Lipids*

Labeled precursors of lipids may include any precursor in lipid biosynthesis.

The precursor molecules of lipids may be CO<sub>2</sub>, NH<sub>3</sub>, glucose, lactate, H<sub>2</sub>O, acetate, and fatty acids.

The precursor may also include labeled water, preferably <sup>2</sup>H<sub>2</sub>O (deuterated water), which is a precursor for fatty acids, glycerol moiety of acyl-glycerols, cholesterol and its derivatives; <sup>13</sup>C or <sup>2</sup>H-labeled fatty acids, which are precursors for triglycerides, phospholipids, cholesterol ester, coamides and other lipids; <sup>13</sup>C- or <sup>2</sup>H-acetate, which is a precursor for fatty acids and cholesterol; <sup>18</sup>O<sub>2</sub>, which is a precursor for fatty acids, cholesterol, acyl-glycerides, and certain oxidatively modified fatty acids (such as peroxides) by either enzymatically catalyzed reactions or by non-enzymatic oxidative damage (e.g. to fatty acids); <sup>13</sup>C- or <sup>2</sup>H-glycerol, which is a precursor for acyl-glycerides; <sup>13</sup>C- or <sup>2</sup>H-labeled acetate, ethanol, ketone bodies or fatty acids, which are precursors for endogenously synthesized fatty acids, cholesterol and acylglycerides; and <sup>2</sup>H or <sup>13</sup>C-labeled cholesterol or its derivatives (including bile acids and steroid hormones). All isotope labeled precursors may be purchased commercially, for example, from Cambridge Isotope Labs (Andover, MA).

Complex lipids, such as glycolipids and cerebroside, can also be labeled from precursors, including <sup>2</sup>H<sub>2</sub>O, which is a precursor for the sugar-moiety of cerebroside (including, but not limited to, N-acetylgalactosamine, N-acetylglucosamine-sulfate, glucuronic acid, and glucuronic acid-sulfate), the fatty acyl-moiety of cerebroside and the sphingosine moiety of cerebroside; <sup>2</sup>H- or <sup>13</sup>C-labeled fatty acids, which are precursors for the fatty acyl moiety of cerebroside, glycolipids and other derivatives.

The precursor molecule may be or include components of lipids.

#### *Precursors of Glycosaminoglycans and Proteoglycans*

Glycosaminoglycans and proteoglycans are a complex class of biomolecules that play important roles in the extracellular space (e.g. cartilage, ground substance, and synovial joint fluid). Molecules in these classes include, for example, the large polymers built from glycosaminoglycans disaccharides, such as hyaluronan, which is a polymer composed of up to 50,000 repeating units of hyaluronic acid (HA) disaccharide, a dimer that contains N-acetyl-glucosamine linked to glucuronic acid; chondroitin-sulfate (CS) polymers, which are built from repeating units of CS disaccharide, a dimer that contains N-acetyl-galactosamine-sulfate linked to glucuronic acid, heparan-sulfate polymers, which are built from repeating units of heparan-sulfate, a dimer of N-acetyl (or N-sulfo)-glucosamine-sulfate linked to glucuronic acid; and keratan-sulfate polymers, which are built from repeating units of keratan-sulfate disaccharide, a dimer that contains N-acetylglucosamine-sulfate linked to galactose. Proteoglycans contain additional proteins that are bound to a central hyaluronan in polymer and other glycosaminoglycans, such as CS, that branch off of the central hyaluronan chain.

Labeled precursors of glycosaminoglycans and proteoglycans include, but are not limited to,  $^2\text{H}_2\text{O}$  (incorporated into the sugar moieties, including N-acetylglucosamine, N-acetylgalactosamine, glucuronic acid, the various sulfates of N-acetylglucosamine and N-acetylgalactosamine, galactose, iduronic acid, and others),  $^{13}\text{C}$ - or  $^2\text{H}$ -glucose (incorporated into said sugar moieties),  $^2\text{H}$ - or  $^{13}\text{C}$ -fructose (incorporated into said sugar moieties),  $^2\text{H}$ - or  $^{13}\text{C}$ -galactose (incorporated into said sugar moieties),  $^{15}\text{N}$ -glycine, other  $^{15}\text{N}$ -labeled amino acids, or  $^{15}\text{N}$ -urea (incorporated into the nitrogen-moiety of said amino sugars, such as N-acetylglucosamine, N-acetyl-galactosamine, etc.);  $^{13}\text{C}$ - or  $^2\text{H}$ -fatty acids,  $^{13}\text{C}$ - or  $^2\text{H}$ -ketone bodies,  $^{13}\text{C}$ -glucose,  $^{13}\text{C}$ -fructose,  $^{18}\text{O}_2$ ,  $^{13}\text{C}$ - or  $^2\text{H}$ -acetate (incorporated into the acetyl moiety of N-acetyl-sugars, such as N-acetyl-glucosamine or N-acetyl-galactosamine), and  $^{18}\text{O}$  or  $^{35}\text{S}$ -labeled sulfate (incorporated into the sulfate moiety of chondroitin-sulfate, heparan-sulfate, keratan-sulfate, and other sulfate moieties). All isotope labeled

precursors may be purchased commercially, for example, from Cambridge Isotope Labs (Andover, MA).

### *Precursors of Carbohydrates*

Labeled precursors of carbohydrates may include any precursor of carbohydrate biosynthesis known in the art. These precursor molecules include but are not limited to H<sub>2</sub>O, monosaccharides (including glucose, galactose, mannose, fucose, glucuronic acid, glucosamine and its derivatives, galactosamine and its derivatives, iduronic acid, fructose, ribose, deoxyribose, sialic acid, erythrose, sorbitol, adols, and polyols), fatty acids, acetate, ketone bodies, ethanol, lactate, alanine, serine, glutamine and other glucogenic amino acids, glycerol, O<sub>2</sub>, CO<sub>2</sub>, urea, starches, disaccharides (including sucrose, lactose, and others), glucose polymers and other polymers of said monosaccharides (including complex polysaccharides).

The precursor molecule may include labeled water, preferably <sup>2</sup>H<sub>2</sub>O, which is a precursor to said monosaccharides, <sup>13</sup>C-labeled glucogenic precursors (including glycerol, CO<sub>2</sub>, glucogenic amino acids, lactate, ethanol, acetate, ketone bodies and fatty acids), <sup>13</sup>C- or <sup>2</sup>H-labeled said monosaccharides, <sup>13</sup>C- or <sup>2</sup>H-labeled starches or disaccharides; other components of carbohydrates labeled with <sup>2</sup>H or <sup>13</sup>C; and <sup>18</sup>O<sub>2</sub>, which is a precursor to monosaccharides and complex polysaccharides.

### *3. Methods of Administering labeled precursor molecules*

Labeled precursors can be administered to an individual by various in vivo methods including, but not limited to, orally, parenterally, subcutaneously, intravenously, and intraperitoneally.

The individual may be an animal. The individual also may be human.

By way of example, in one embodiment, the labeled precursor is <sup>2</sup>H<sub>2</sub>O that can be ingested (e.g., by drinking or intravenous infusion) by an individual. In another embodiment, the labeled precursor is <sup>13</sup>C<sub>1</sub>-lysine that can be ingested (e.g., by drinking or intravenous infusion) by an individual. In another embodiment, the

labeled precursor is  $^{13}\text{C}_1$ -glycine that can be ingested (*e.g.*, by drinking or intravenous infusion) by an individual. In another embodiment, the labeled precursor is  $^2\text{H}_3$ -leucine that can be ingested (*e.g.*, by drinking or intravenous infusion) by an individual. In another embodiment, the labeled precursor is  $^2\text{H}_2$ -glucose that can be ingested (*e.g.* by drinking or intravenous infusion) by an individual.

The length of time for which the labeled precursor is administered may be sufficient to allow the precursor molecule to become incorporated into a biosynthetic pathway. The isotope-labeled precursor molecule also may be introduced to an individual for a period of time sufficient for the label of said isotope-labeled precursor molecule to become incorporated into one or more biological molecules and then released in the form of one or more labeled and unlabeled metabolic derivatives of the one or more biological molecules. The period of time may be a pre-determined length of time. This required duration of time may range from minutes or hours (*e.g.*, for fast turnover biological molecules), to weeks or even months (*e.g.*, for slow-turnover biological molecules).

The precursor molecule may be continuously or repeatedly administered. Administration of the precursor can be achieved in various ways. The precursor molecule may be administered continuously or repeatedly, so that a sufficient amount of precursor is administered such that an isotopic plateau value of maximal or isotopic enrichment is approached (*i.e.* wherein the concentration of labeled precursor is relatively constant over time). For example, see Figure 7. If the continuous labeling period can be maintained for as long as 4-5 half-lives of a biological molecule, the asymptote reached and the shape of the isotope enrichment curve approaching this asymptote will reveal the "true precursor" isotopic enrichment as well as the fractional replacement rate of the biological molecule product (Figure 7). By labeling to plateau while maintaining a stable precursor pool enrichment, it is thereby possible to overcome the biological complexities of cellular metabolite pools.

The precursor molecule may be administered discontinuously. For the discontinuous labeling method, an amount of labeled precursor molecule is measured and then administered, one or more times, and then the exposure to labeled precursor molecule is discontinued and wash-out of labeled precursor molecule from body precursor pool is allowed to occur. The time course of biological molecule breakdown may then be monitored by measurement of the loss of label or decay of label incorporation (dilution or die-away) in the metabolic derivative of the biological sample.

### *B. Biological Molecules and Metabolic Derivatives*

In one aspect, the invention includes a method for determining the rate of synthesis or breakdown of a biological molecule in an individual by detecting the incorporation of isotope label in metabolic derivatives of the biological molecule.

Preferably, the biological molecules are inaccessible biological molecules or are found in inaccessible biological samples. The rate of biosynthesis or breakdown of the inaccessible biological molecules may be measured by measuring isotope labeling or decay in metabolic derivatives or catabolic products.

Moreover, the metabolic derivatives or catabolic products of the biological molecules are preferably in an accessible biological sample. The metabolic derivatives or catabolic products also preferably derive primarily, and optionally uniquely, from the biological molecule. Thus, the metabolic derivatives or catabolic products preferably identify or characterize, the biological molecule, and are thus said to be identifiers of the biological molecule. Preferably, only small quantities, and not the total quantity, of metabolic derivatives or catabolic products need to be acquired. Further, the metabolic derivatives or catabolic products preferably cannot be re-incorporated into other biological molecules via metabolism.

Representative precursor molecules, inaccessible biological molecules, and metabolic derivatives are depicted in Table 1. The precursor molecule may be

incorporated via biosynthesis into a biological molecule and subsequent breakdown to form one or more metabolic derivatives. Metabolic derivatives incorporating the label may then be correlated to the biological molecule from which they were derived, and the label incorporation or decay kinetics in the metabolic derivative may reveal the label incorporation or decay kinetics in the biological molecule from which they were derived.

**Table 1: Precursors, Inaccessible Biological Molecules, and Metabolic Derivatives**

Precursor Molecule	Inaccessible Biological Molecule	Metabolic Derivatives
<sup>13</sup> C-lysine <sup>15</sup> N-histidine <sup>13</sup> C-serine <sup>13</sup> C-glycine <sup>2</sup> H <sub>5</sub> -histidine Other deuterated, <sup>15</sup> N-labeled or <sup>13</sup> C-labeled amino acids Labeled Water <sup>18</sup> O <sub>2</sub>	Bone Collagen	Pyridinoline Hydroxy-pyridinoline N- and C-terminal telopeptides and propeptides 4-hydroxyproline 3-hydroxyproline hydroxylysine glucosylgalactosyl- hydroxylysine galactosylhydroxylysine
<sup>13</sup> C-lysine <sup>15</sup> N-histidine <sup>13</sup> C-serine <sup>13</sup> C-glycine <sup>2</sup> H <sub>5</sub> -histidine Other deuterated, <sup>15</sup> N-labeled or <sup>13</sup> C-labeled amino acids Labeled Water <sup>18</sup> O <sub>2</sub>	Cardiac Collagen	Pyridinoline Hydroxy-pyridinoline N- and C-terminal telopeptides and propeptides 4-hydroxyproline 3-hydroxyproline hydroxylysine glucosylgalactosyl- hydroxylysine galactosylhydroxylysine
<sup>13</sup> C-lysine <sup>15</sup> N-histidine <sup>13</sup> C-serine <sup>13</sup> C-glycine <sup>2</sup> H <sub>5</sub> -histidine Other deuterated, <sup>15</sup> N-labeled or <sup>13</sup> C-labeled amino acids Labeled Water <sup>18</sup> O <sub>2</sub>	Liver Collagen	Pyridinoline Hydroxy-pyridinoline N- and C-terminal telopeptides and propeptides 4-hydroxyproline 3-hydroxyproline hydroxylysine glucosylgalactosyl- hydroxylysine galactosylhydroxylysine

Precursor Molecule	Inaccessible Biological Molecule	Metabolic Derivatives
<sup>13</sup> C-lysine <sup>15</sup> N-histidine <sup>13</sup> C-serine <sup>13</sup> C-glycine <sup>2</sup> H <sub>5</sub> -histidine Other deuterated, <sup>15</sup> N-labeled or <sup>13</sup> C-labeled amino acids Labeled Water <sup>18</sup> O <sub>2</sub>	Lung Collagen	Pyridinoline Hydroxy-pyridinoline N- and C-terminal telopeptides and propeptides 4-hydroxyproline 3-hydroxyproline hydroxylysine glucosylgalactosyl- hydroxylysine galactosylhydroxylysine
<sup>13</sup> C-lysine <sup>15</sup> N-histidine <sup>13</sup> C-serine <sup>13</sup> C-glycine <sup>2</sup> H <sub>5</sub> -histidine Other deuterated, <sup>15</sup> N-labeled or <sup>13</sup> C-labeled amino acids Labeled Water <sup>18</sup> O <sub>2</sub>	Skin Collagen	Pyridinoline Hydroxy-pyridinoline N- and C-terminal telopeptides and propeptides 4-hydroxyproline 3-hydroxyproline hydroxylysine glucosylgalactosyl- hydroxylysine galactosylhydroxylysine
<sup>13</sup> C-lysine <sup>15</sup> N-histidine <sup>13</sup> C-serine <sup>13</sup> C-glycine <sup>2</sup> H <sub>5</sub> -histidine Other deuterated, <sup>15</sup> N-labeled or <sup>13</sup> C-labeled amino acids Labeled Water <sup>18</sup> O <sub>2</sub>	Brain Amyloid Precursor Protein, Brain Amyloid Fibrils	A-beta (1-40) (SEQ ID NO:27) A-beta (1-42) (SEQ ID NO:28) Amyloid precursor protein C-peptide
<sup>13</sup> C-lysine <sup>15</sup> N-histidine <sup>13</sup> C-histidine <sup>13</sup> C-serine <sup>13</sup> C-glycine <sup>2</sup> H <sub>5</sub> -histidine Other deuterated, <sup>15</sup> N-labeled or <sup>13</sup> C-labeled amino acids Labeled Water <sup>18</sup> O <sub>2</sub>	Muscle Myosin	3-methyl-histidine Peptides from myosin or actin



Precursor Molecule	Inaccessible Biological Molecule	Metabolic Derivatives
<sup>13</sup> C-lysine <sup>15</sup> N-histidine <sup>13</sup> C-serine <sup>13</sup> C-glycine <sup>2</sup> H <sub>5</sub> -histidine Other deuterated, <sup>15</sup> N-labeled or <sup>13</sup> C-labeled amino acids Labeled Water	Myelin basic protein(MBP)	Brain MBP-like material
<sup>15</sup> N-histidine <sup>13</sup> C-lysine <sup>13</sup> C-serine <sup>13</sup> C-glycine <sup>2</sup> H <sub>5</sub> -histidine Other deuterated, <sup>15</sup> N-labeled or <sup>13</sup> C-labeled amino acids Labeled Water	Prostate-specific antigen (PSA) Prostate-specific membrane antigen (PSMA)	Peptides from PSA or PSMA
<sup>13</sup> C-lysine <sup>15</sup> N-histidine <sup>2</sup> H <sub>5</sub> -histidine Other deuterated, <sup>15</sup> N-labeled or <sup>13</sup> C-labeled amino acids Labeled water	Polynucleotides (DNA or RNA), methylated or oxidized nucleotides	Deoxycytosine methyl-deoxycytosine 8-oxo-guanine Ribose Deoxyribose
Labeled water <sup>13</sup> C-Acetate or <sup>13</sup> C-ethanol <sup>2</sup> H- or <sup>13</sup> C- fatty acids <sup>2</sup> H- or <sup>13</sup> C-ketone bodies <sup>18</sup> O <sub>2</sub> <sup>2</sup> H- or <sup>13</sup> C-labeled cholesterol	Brain membrane cholesterol	24-(S)-hydroxycholesterol
Labeled Water <sup>18</sup> O <sub>2</sub> <sup>13</sup> C-acetate <sup>2</sup> H- or <sup>13</sup> C-glucose <sup>2</sup> H- or <sup>13</sup> C-galactose <sup>13</sup> C-serine <sup>2</sup> H or <sup>13</sup> C-fatty acids <sup>13</sup> C-alanine <sup>13</sup> C-lactate	Brain myelin lipids	Galactosyl-cereboside Sphingomyelin Sphingosines

Precursor Molecule	Inaccessible Biological Molecule	Metabolic Derivatives
Labeled Water $^{18}\text{O}_2$ $^{13}\text{C}$ -acetate $^2\text{H}$ - or $^{13}\text{C}$ -glucose $^2\text{H}$ - or $^{13}\text{C}$ -galactose $^{13}\text{C}$ -serine $^2\text{H}$ or $^{13}\text{C}$ -fatty acids	Pancreatic $\beta$ -cell membrane lipids	Circulating $\beta$ -cell specific membrane lipids
$^{13}\text{C}$ -lysine $^{15}\text{N}$ -histidine $^2\text{H}_5$ -histidine Other deuterated, $^{15}\text{N}$ -labeled or $^{13}\text{C}$ -labeled amino acids Labeled Water $^{18}\text{O}_2$	Pancreatic $\beta$ -cell proteins	Insulin C-peptide Islet amyloid protein
Labeled Water $^{13}\text{C}$ -acetate or $^{13}\text{C}$ -ethanol $^2\text{H}$ - or $^{13}\text{C}$ -fatty acids $^2\text{H}$ - or $^{13}\text{C}$ -ketone bodies $^{18}\text{O}_2$ $^2\text{H}$ - or $^{13}\text{C}$ -labeled cholesterol	Tissue cholesterol (hepatic, adrenal, ovarian, testicular)	Bile acids Steroid hormones
Labeled water $^2\text{H}$ - or $^{13}\text{C}$ -glucose $^2\text{H}$ - or $^{13}\text{C}$ -galactose $^2\text{H}$ - or $^{13}\text{C}$ -glucosamine $^{13}\text{C}$ -alanine $^{13}\text{C}$ -lactate	Synovial fluid hyaluronan, glycosaminoglycans, or proteoglycans	Hyaluronic acid disaccharide or polymers; N-acetyl glucosamine, N-acetyl-galactosamine, chondroitin-sulfate disaccharide or polymers; Heparin sulfate disaccharide or polymers
Labeled water $^2\text{H}$ - or $^{13}\text{C}$ -glucose $^2\text{H}$ - or $^{13}\text{C}$ -galactose $^2\text{H}$ - or $^{13}\text{C}$ -glucosamine $^{13}\text{C}$ -alanine $^{13}\text{C}$ -lactate	Cartilage hyaluronan, glycosaminoglycans, or proteoglycans	Hyaluronic acid disaccharide or polymers Chondroitin-sulfate disaccharide or polymers Heparin-sulfate disaccharide or polymers N-Acetyl-glucosamine, N-acetyl-galactosamine

The metabolic derivative may be obtained in an accessible biological sample.

The metabolic derivative is acquired in quantities that are sufficient for performing

isotopic measurements, MIDA, and calculations of the proportions of isotopically labeled:unlabeled species.

#### *Proteins and their Metabolic Derivatives*

The biological molecules may be proteins. Examples of proteins are listed in Table 1, including collagen, myosin, and amyloid precursor protein.

One or more metabolic derivatives may be produced during biosynthesis or breakdown of the proteins. The metabolic derivatives of proteins may be amino acids and peptides. The metabolic derivatives may also be portions of the amino acids and peptides.

Preferably, the proteins are inaccessible biological molecules in inaccessible biological samples. Acquiring inaccessible proteins requires invasive procedures involving substantial risk and discomfort. The rate of biosynthesis or breakdown of the collagens is preferably measured by measuring metabolic derivatives of proteins in accessible biological samples.

The metabolic derivatives of preferably are preferably in an accessible biological sample. The metabolic derivatives of proteins preferably derive primarily, and optionally uniquely, from the specific types of collagen. Thus, protein metabolic derivatives or catabolic products preferably identify or characterize types of collagens, and their tissue source. Only small quantities, and not the total quantity, of collagen metabolic derivatives or catabolic products need to be acquired. Further, the metabolic derivatives or catabolic products of collagen cannot be directly re-incorporated into collagens or other biological molecules.

Metabolic derivatives may include one or more post-translational modifications. In one embodiment, the metabolic derivative may include, but is not limited to, a phosphorylated, methylated, hydroxylated, glycosylated, N-acetyl-glucosaminated, prenylated, palmitoylated, gamma-carboxylated, acetylated, sulfated, or other post-translationally modified amino acid or peptide wherein the peptide's composition or the amino acid's structure uniquely identifies the biological protein from which it is

derived. Examples of this type of metabolic derivative (and the proteins from which they were derived) include 3-methyl-histidine (muscle myosin), hydroxyproline, hydroxylysine, glucosylgalactosyl-hydroxylysine, galactosylhydroxylysine (collagen) and gamma-carboxyglutamate (collagen).

### *Collagen*

The biological molecule may be collagen and the metabolic derivative is an identifier of collagen. Biosynthesis and breakdown of collagen has been implicated in osteoporosis, fibrogenic disorders (e.g. hepatic cirrhosis, congestive heart failure, fibrotic lung disease, and photo-aging) rheumatoid arthritis, diabetes mellitus, and several kinds of cancers and disorders relating to unregulated cell growth.

Collagen is a triple stranded helical protein having 3 separate polypeptides, called tropocollagen. Collagen synthesized first as three separate strands of procollagen. Procollagen forms a triple helix. The N-terminal and C-terminal peptides of procollagen are cleaved to produce tropocollagen. Collagen may be cross-linked.

Collagens are inaccessible biological molecules in inaccessible biological samples. Acquiring collagen samples directly from bone, for example, is an invasive procedure requiring substantial risk and discomfort. The rate of biosynthesis or breakdown of the collagens is preferably measured by measuring metabolic derivatives of collagens in accessible biological samples.

The metabolic derivatives of collagens are preferably in an accessible biological sample. The metabolic derivatives of collagen preferably derive primarily, and optionally uniquely, from the specific types of collagen. For example, N- and C-terminal collagen telopeptides, N- and C-terminal collagen propeptides, and post-translational modifications of collagens derive primarily from specific collagen types. Thus, the collagen metabolic derivatives or catabolic products preferably identify or characterize types of collagens, and their tissue source. Only small quantities, and not the total quantity, of collagen metabolic derivatives or catabolic products need to

be acquired. Further, the metabolic derivatives or catabolic products of collagen cannot be directly re-incorporated into collagens or other biological molecules.

Collagens are classified into several different types.

Type I collagen is one of the most abundant protein species in the human body, accounting for at least 70% of total collagens. Most of this is present in bones, where about 90% of the organic matrix consists of type I collagen. The remainder is found in soft connective tissues all over the body, including hepatic tissue, cardiac tissue, lung tissue, and skin. The type I collagen molecule is a long, rigid rod - a shape necessary for its function as part of the collagen fiber in tissue. Two of the three constituent chains of the normal type I collagen molecule are identical  $\alpha 1(I)$  chains, while the third is a different but homologous  $\alpha 2(I)$  chain. These chains are all intertwined into a triple helix. The original gene products, the pro- $\alpha 1(I)$  and pro- $\alpha 2(I)$  of type I procollagen, are about 50% longer than the corresponding final products,  $\alpha$  chains. The two additional, bulky domains at both ends of the molecule are usually called the amino-terminal and the carboxy-terminal propeptide of type I procollagen. These parts are removed en bloc from the procollagen by two specific endoproteinases, the N- and C-proteinases, once the molecule has reached the extracellular space.

Type II collagen is the major fibrous collagen of cartilage, representing 80-90% of the collagen in this tissue. Type II collagen is produced by chondrocytes, and its fibers make up 40-50% of cartilage dry weight. It is closely linked with type XI collagen, with which it has striking sequence homology. The globular domains of type XI and the increased glycosylation of type II collagen compared with the types I and III may have a role in the determination of the fibril diameter. The major function of type II collagen is to provide the tensile strength and toughness of cartilage.

The main cells synthesizing type I collagen in soft tissues are fibroblasts, which also always produce significant amounts of type III collagen. Type III collagen is the second most abundant collagen type in the human body. Its thin fibrils constitute the

principal collagen in blood vessels and, together with type I collagen, in newly formed soft connective tissue. Its relative concentration is particularly large in young, metabolically active connective tissue, e.g. the granulation tissue of a healing wound. During wound healing its proportion decreases, probably due to the half-life, which is shorter for type III collagen than for type I collagen. The type III collagen molecule is a homotrimer of three identical  $\alpha 1$ (III) chains. Its fibres are generally thinner than those containing mainly type I collagen and these fibers are covered by type III pN-collagen with retained aminoterminal propeptide. Such molecules are believed to prevent further lateral growth of the fiber.

Type IV collagen is a network forming collagen. Type IV collagens assemble into a feltlike sheet or meshwork that constitutes a major part of mature basal laminae.

Representative metabolic derivatives of collagen are listed in Table 1. The metabolic derivative may be an N- or C- terminal telopeptide or an N- or C-terminal propeptide, including but not limited to N-terminal telopeptide  $\alpha$ (I) (SEQ ID NO:1), N-terminal telopeptide  $\alpha 2$ (I) (SEQ ID NO:2), N-terminal telopeptide  $\alpha 2$ (I) (SEQ ID NO:3), N-terminal telopeptide  $\alpha 1$ (II) (SEQ ID NO:4), N-terminal telopeptide  $\alpha 1$ (III) (SEQ ID NO:5), C-terminal telopeptide  $\alpha 1$ (I) (SEQ ID NO:6), C-terminal telopeptide  $\alpha 2$ (I) (SEQ ID NO:7), C-terminal telopeptide  $\alpha 1$ (II) (SEQ ID NO:8), C-terminal telopeptide  $\alpha 1$ (II) (SEQ ID NO:9), C-terminal telopeptide  $\alpha 1$ (II) (SEQ ID NO:10), C-terminal telopeptide  $\alpha 1$ (III) (SEQ ID NO:11), cross-linked carboxy-terminal peptide of type I collagen (ICTP), PINP( $\alpha 1$ ) (SEQ ID NO:12), PICP( $\alpha 1$ ) (SEQ ID NO:13), PINP( $\alpha 2$ ) (SEQ ID NO:14), PICP( $\alpha 2$ ) (SEQ ID NO:15), PIINP( $\alpha 1$ ) (SEQ ID NO:16), PIICP( $\alpha 1$ ) (SEQ ID NO:17), PIINP( $\alpha 1$ ) (SEQ ID NO:18), PIICP( $\alpha 1$ )(SEQ ID NO:19), PIVNP( $\alpha 1$ )(SEQ ID NO:20), PIVNP( $\alpha 2$ )(SEQ ID NO:21), PIVNP( $\alpha 2$ )(SEQ ID NO:22), PIVNP( $\alpha 3$ ) (SEQ ID NO:23), PIVNP( $\alpha 4$ ) (SEQ ID NO:24), PIVNP( $\alpha 5$ ) (SEQ ID NO:25), and PIVNP( $\alpha 6$ ) (SEQ ID NO:26). Each peptide identifies a specific type of collagen.

The collagen metabolic derivative is specific to the source of collagen. Table 1 shows specific collagen products associated with each tissue. The metabolic derivative also may be a post-translational modification or crosslink of collagen. Post-translational modifications of collagen include pyridinoline, hydroxy-pyridinoline, 4-hydroxyproline, 3-hydroxyproline, hydroxylysine, glucosylgalactosyl-hydroxylysine, and galactosylhydroxylysine. Each of these metabolic derivatives is an identifier of collagen.

One skilled in the art will recognize that other known metabolic derivatives of collagen may be detected by the methods described herein.

### *Myosin*

The biological molecule may also be myosin from muscle tissue. Muscle is a muscle protein that drives muscle contraction by binding actin and hydrolyzing ATP. Myosin biosynthesis and breakdown may be determined by identifying myosin breakdown products.

Myosin is an inaccessible biological molecule found in inaccessible biological samples. Acquiring myosin samples directly in muscle tissue biopsies, for example, is an invasive procedure requiring substantial risk and discomfort. The rate of biosynthesis or breakdown of myosin is preferably determined by measuring metabolic derivatives of myosin in accessible biological samples.

The metabolic derivatives of myosin are preferably in an accessible biological sample. The metabolic derivatives of myosin preferably derive primarily, and optionally uniquely, from the myosin. 3-methyl histidine, for example, derives primarily from myosin. Peptides released from myosin during proteolysis may escape into the circulation and identify myosin. Thus, the myosin metabolic derivatives or catabolic products preferably identify or characterize myosin. Only small quantities, and not the total quantity, of myosin metabolic derivatives or catabolic products need to be acquired. Optionally, the metabolic derivatives or

catabolic products of myosin cannot be directly re-incorporated into myosin or other biological molecules.

Table 1 lists examples of metabolic derivatives specific to myosin.

One skilled in the art will recognize that other known metabolic derivatives of myosin may be detected by the methods described herein.

#### *Amyloid Precursor Protein*

The biological molecule may be Amyloid Precursor Protein (APP). The identification of amyloid-rich plaques has long been a diagnostic tool for pathologists investigating Alzheimer's disease. The plaques are formed through the accumulation and aggregation of beta-amyloid peptides derived from the APP, and are characteristically found in the brain parenchyma and around blood vessels.

APP is an inaccessible biological molecule found in inaccessible biological samples. Acquiring APP samples directly from brain, for example, is an invasive procedure requiring substantial risk or resulting in serious injury or death. The rate of biosynthesis or breakdown of APP is preferably determined by measuring metabolic derivatives of APP in accessible biological samples.

The metabolic derivatives of APP are preferably in an accessible biological sample. The metabolic derivatives of APP preferably derive primarily, and optionally uniquely, from the APP. Beta amyloid precursor peptides, for example, derive specifically from APP. Thus, the APP metabolic derivatives or catabolic products preferably identify or characterize APP. Only small quantities, and not the total quantity, of APP metabolic derivatives or catabolic products need to be acquired. Further, the metabolic derivatives or catabolic products of APP cannot be directly re-incorporated into APP or other biological molecules.

The metabolic derivative may be an APP specific metabolic derivative. The metabolic derivative may be amyloid beta (1-40) (SEQ ID NO:27), amyloid beta (1-42) (SEQ ID NO:28), or APP C peptide. One of skill in the art will recognize that the



metabolic derivatives may be components of amyloid beta (1-40) (SEQ ID NO:27), amyloid beta (1-42) (SEQ ID NO:28), or other APP specific metabolic derivatives.

One skilled in the art will recognize that other known metabolic derivatives of APP may be detected by the methods described herein.

#### *Myelin Basic Protein*

The biological molecule may be myelin basic protein (MBP). Loss of MBP, or demyelination, is associated with multiple sclerosis, a neurodegenerative disease. Increase in MBP-like material in urine is associated with demyelination.

MBP is an inaccessible biological molecule found in inaccessible biological samples. Acquiring MBP samples directly from brain, for example, is an invasive procedure carrying a risk of serious injury or death. Preferably, the rate of biosynthesis or breakdown of MBP determined by measuring metabolic derivatives of MBP in accessible biological samples.

The metabolic derivatives of MBP are preferably in an accessible biological sample. The metabolic derivatives of MBP preferably derive primarily, and optionally uniquely, from the MBP. MBP-like material, for example, derives primarily from MBP. Thus, the MBP metabolic derivatives or catabolic products identify or characterize MBP. Only small quantities, and not the total quantity, of MBP metabolic derivatives or catabolic products need to be acquired. Further, the metabolic derivatives or catabolic products of MBP cannot be directly re-incorporated into MBP or other biological molecules.

The metabolic derivative may be specific to MBP. In one embodiment, the catalytic product is MBP-like material. In a further embodiment, the MBP-like material is in urine.

One skilled in the art will recognize that other known metabolic derivatives of MBP may be detected by the methods described herein.

### *Polynucleotides and their Metabolic Derivatives*

The biological molecule may also be polynucleotide. The polynucleotide may be DNA or RNA.

DNA biosynthesis and breakdown are associated with cell proliferation and death, respectively. Cancer and other disorders relating to cell proliferation may be monitored determining the rate of biosynthesis and breakdown of polynucleotides.

Preferably, polynucleotides are inaccessible biological molecules in inaccessible biological samples. The rate of biosynthesis or breakdown of the polynucleotides is preferably measured by measuring metabolic derivatives of polynucleotides in accessible biological samples.

The metabolic derivatives of polynucleotides are preferably in an accessible biological sample. The metabolic derivatives of polynucleotides preferably derive primarily, and optionally uniquely, from polynucleotides. The metabolic derivatives or catabolic products preferably identify or characterize polynucleotides, and optionally their tissue source. Only small quantities, and not the total quantity, of metabolic derivatives or catabolic products need to be acquired. Further, the metabolic derivatives or catabolic products cannot be directly re-incorporated into polynucleotides or other biological molecules.

Table 1 lists examples of metabolic derivatives from polynucleotides.

The metabolic derivative may be a DNA- or RNA-specific metabolic derivative such as deoxyribose, ribose, or a specific sequence of polynucleotides. The metabolic derivative may be produced by post-replication modification of bases in DNA. In another embodiment, the metabolic derivative is a methylated or oxidatively modified base. In another embodiment, the metabolic derivative may be a methyl-cytosine, 8-oxo-guanosine, deoxyribose, and ribose.

One skilled in the art will recognize that other known metabolic derivatives of polynucleotides may be detected by the methods described herein.

### *Lipids and their Metabolic Derivatives*

The biological molecule also may be a lipid. Lipids are components of membranes, including membranes in brain, pancreas and other tissues. Lipids include, but are not limited to, acyl-glycerides, phospholipids, cholesterol and its derivatives, ceramides, sphingosines, and glycolipids.

Frequently, lipids are an inaccessible biological molecules found in inaccessible biological samples. Acquiring lipid samples directly from brain, for example, is an invasive procedure requiring substantial risk and discomfort. The rate of biosynthesis or breakdown of lipids is preferably measured by measuring metabolic derivatives of lipids in accessible biological samples.

The metabolic derivatives of lipids are preferably in an accessible biological sample. 22-(R)-hydroxycholesterol, 24-(S)-hydroxycholesterol, or 24,25-(S)-epoxycholesterol, galactocerebroside, galactose from galactocerebroside, sphingomyelin, and sphingosines for example, specifically identify their lipids of origin. The metabolic derivatives of lipids preferably derive primarily, and optionally uniquely, from the lipids. Thus, the lipid metabolic derivatives or catabolic products preferably identify or characterize lipids. Only small quantities, and not the total quantity, of lipid metabolic derivatives or catabolic products need to be acquired. Further, the metabolic derivatives or catabolic products of lipids preferably cannot be directly re-incorporated into lipids or other biological molecules.

#### *Glycosaminoglycans, Proteoglycans, and their Metabolic Derivatives*

Glycosaminoglycans and proteoglycans are a complex class of biomolecules that play important roles in the extracellular space (e.g. cartilage, ground substance, and synovial joint fluid).

Preferably, the glycosaminoglycans and proteoglycans are inaccessible biological molecules in inaccessible biological samples. The rate of biosynthesis or breakdown of the polynucleotides is preferably measured by measuring metabolic derivatives of glycosaminoglycans and proteoglycans in accessible biological samples.

The metabolic derivatives of glycosaminoglycans and proteoglycans are preferably in an accessible biological sample. The metabolic derivatives of glycosaminoglycans and proteoglycans preferably derive primarily, and optionally uniquely, from glycosaminoglycans or proteoglycans. The metabolic derivatives or catabolic products preferably identify or characterize glycosaminoglycans and proteoglycans, and optionally their tissue source. Only small quantities, and not the total quantity, of metabolic derivatives or catabolic products need to be acquired. Further, the metabolic derivatives or catabolic products cannot be directly re-incorporated into glycosaminoglycans, proteoglycans or other biological molecules.

The metabolic derivative may include one or more of the following: hyaluronic acid disaccharide or polymers thereof, N-acetyl glucosamine, N-acetyl-galactosamine, chondroitin-sulfate disaccharide or polymers thereof, heparin sulfate disaccharide or polymers thereof, and keratin sulfate disaccharide or polymers thereof.

The metabolic derivatives of lipids are preferably in an accessible biological sample. Hyaluronic acid disaccharide or polymers thereof, N-acetyl glucosamine, N-acetyl-galactosamine, chondroitin-sulfate disaccharide or polymers thereof, and Heparin sulfate disaccharide or polymers thereof, for example, specifically identify their origin. The metabolic derivatives or catabolic products preferably identify or characterize specific glycosaminoglycans or proteoglycans.

*(ii) obtaining one or more biological samples from said individual*

Biological samples are obtained from the individual. Specific methods of obtaining biological samples are well known in the art. Preferably, the biological sample is an accessible biological sample.

Biosynthesis or breakdown of the biological molecule may occur at a different tissue or fluid from the obtained one or more biological samples.

One or more metabolic derivatives may be obtained, and optionally partially purified or isolated, from the biological sample using standard biochemical methods known in the art.

The frequency of biological sampling can vary depending on different factors. Such factors include, but are not limited to, the nature of the metabolic derivatives, ease and safety of sampling, biological rate constants and turnover kinetics of the metabolic derivative or the biological molecule from which it was derived, and the half-life of a drug used in a treatment if monitoring responses to treatment.

The one or more metabolic derivatives may also be purified partially purified, or optionally, isolated, by conventional purification methods including high pressure liquid chromatography (HPLC), fast performance liquid chromatography (FPLC), chemical extraction, thin layer chromatography, gas chromatography, gel electrophoresis, and/or other separation methods known to those skilled in the art.

In another embodiment, the one or more metabolic derivatives may be hydrolyzed or otherwise degraded to form smaller molecules. Hydrolysis methods include any method known in the art, including, but not limited to, chemical hydrolysis (such as acid hydrolysis) and biochemical hydrolysis (such as peptidase or nuclease degradation). Hydrolysis or degradation may be conducted either before or after purification and/or isolation of the metabolic derivative. The metabolic derivatives also may be partially purified, or optionally, isolated, by conventional purification methods including high performance liquid chromatography (HPLC), fast performance liquid chromatography (FPLC), gas chromatography, gel electrophoresis, and/or any other methods of separating chemical and/or biochemical compounds known to those skilled in the art.

*iii) Detecting the incorporation of said label in said one or more metabolic derivatives*

Isotopic enrichment in metabolic derivatives can be determined by various methods such as mass spectrometry, including but not limited to gas chromatography-

mass spectrometry (GC-MS), isotope-ratio mass spectrometry, GC-isotope ratio-combustion-MS, GC-isotope ratio-pyrolysis-MS, liquid chromatography-MS, electrospray ionization-MS, matrix assisted laser desorption-time of flight-MS, Fourier-transform-ion-cyclotron-resonance-MS, cycloidal-MS, nuclear magnetic resonance (NMR), or liquid scintillation counting.

Incorporation of labeled isotopes into biological molecules may be measured directly. Alternatively, incorporation of labeled isotopes may be determined by measuring the incorporation of labeled isotopes into one or metabolic derivatives, or hydrolysis or degradation products of metabolic derivatives. The hydrolysis products may optionally be measured following either partial purification or isolation by any known separation method, as described previously.

*a. Mass Spectrometry*

Mass spectrometers convert components of a sample into rapidly moving gaseous ions and separate them on the basis of their mass-to-charge ratios. The distributions of isotopes or isotopologues of ions, or ion fragments, may thus be used to measure the isotopic enrichment in one or more metabolic derivatives.

Generally, mass spectrometers include an ionization means and a mass analyzer. A number of different types of mass analyzers are known in the art. These include, but are not limited to, magnetic sector analyzers, electrostatic analyzers, quadrupoles, ion traps, time of flight mass analyzers, and fourier transform analyzers. In addition, two or more mass analyzers may be coupled (MS/MS) first to separate precursor ions, then to separate and measure gas phase fragment ions.

Mass spectrometers may also include a number of different ionization methods. These include, but are not limited to, gas phase ionization sources such as electron impact, chemical ionization, and field ionization, as well as desorption sources, such as field desorption, fast atom bombardment, matrix assisted laser desorption/ionization, and surface enhanced laser desorption/ionization.

In addition, mass spectrometers may be coupled to separation means such as gas chromatography (GC) and high performance liquid chromatography (HPLC). In gas-chromatography mass-spectrometry (GC/MS), capillary columns from a gas chromatograph are coupled directly to the mass spectrometer, optionally using a jet separator. In such an application, the gas chromatography (GC) column separates sample components from the sample gas mixture and the separated components are ionized and chemically analyzed in the mass spectrometer.

When GC/MS is used to measure mass isotopomer abundances of organic molecules, hydrogen-labeled isotope incorporation from labeled water is amplified 3 to 7-fold, depending on the number of hydrogen atoms incorporated into the organic molecule from labeled water.

In one embodiment, isotope enrichments of metabolic derivatives may be measured directly by mass spectrometry.

In another embodiment, the metabolic derivatives may be partially purified, or optionally isolated, prior to mass spectral analysis. Furthermore, hydrolysis or degradation products of metabolic derivatives may be purified.

In another embodiment, isotope enrichments of metabolic derivatives after hydrolysis of the metabolic derivative are measured by gas chromatography-mass spectrometry.

In each of the above embodiments the biosynthesis rate of the biological molecule can be calculated by application of the precursor-product relationship using either labeled precursor molecule enrichment values or asymptotic isotope enrichment in the relevant metabolic derivative of a fully turned over biological molecule to represent the true precursor pool enrichment. Alternatively, the biosynthesis or breakdown rate may be calculated using an exponential decay curve by application of exponential or other die-away kinetic models.

*b. Liquid Scintillation*

Radioactive isotopes may be observed using a liquid scintillation counter. Radioactive isotopes such as  $^3\text{H}$  emit radiation that is detected by a liquid scintillation detector. The detector converts the radiation into an electrical signal, which is amplified. Accordingly, the number of radioactive isotopes in a metabolic derivative may be measured.

In one embodiment, the radioisotope-enrichment value in a biological sample may be measured directly by liquid scintillation. In a further embodiment, the radioisotope is  $^3\text{H}$ .

In another embodiment, the metabolic derivative or components thereof may be partially purified, or optionally isolated, and subsequently measured by liquid scintillation counting.

In each of the above embodiments the biosynthesis rate of the biological molecule can be calculated by application of the precursor-product relationship using either labeled precursor molecule enrichment values or asymptotic isotope enrichment in the relevant metabolic derivative of a fully turned over biological molecule to represent the true precursor pool enrichment. Alternatively, the breakdown rate may be calculated using an exponential or other die-away model decay curve.

*(iv) Determining the rate of biosynthesis or breakdown*

Biosynthetic and breakdown rates may be calculated by combinatorial analysis, by hand or via an algorithm. Variations of Mass Isotopomer Distribution Analysis (MIDA) combinatorial algorithm are discussed in a number of different sources known to one skilled in the art. Specifically, the MIDA calculation methods are the subject of U.S. Patent No. 5,336,686, incorporated herein by reference. The method is further discussed by Hellerstein and Neese (1999), as well as Chinkes, et al. (1996), and Kelleher and Masterson (1992).

In addition to the above-cited references, calculation software implementing the method is publicly available from Professor Marc Hellerstein, University of California, Berkeley.



The biosynthesis rate ( $k$ ) of biological molecule may be calculated, using the standard isotope dilution equation, for example

$$A_t = A_0 \cdot e^{-kt},$$

where  $A_t$  = the proportion of labeled metabolic derivative in a sample at time  $t$

$A_0$  = the proportion of labeled metabolic derivative in sample at time zero

$t$  = time

$k$  = biosynthesis rate constant

$$k = \frac{-\ln\left(\frac{A_t}{A_0}\right)}{t}.$$

Similarly, breakdown rate constants may be calculated based on an exponential or other kinetic decay curve, known to those skilled in the art.

#### IV. Methods of Use

The method disclosed herein has many biological and medical applications. The measurements described herein are applicable for numerous medical utilities such as monitoring pre-existing physiological conditions, diagnosis of disease states, and assessing risk of development of disease states or physiological conditions, in addition to pharmaceutical research utilities, such as screening of candidate gene or protein targets, phenotypic validation of candidate drug agents, FDA phase I and II human validation studies of candidate drug agents, FDA phase III approval of candidate drug agents, and FDA phase IV approval studies, or other post approval market positioning or mechanism of drug action studies. Table 2 shows a number of diseases and disorders that correlate to different inaccessible biological molecules.

In one aspect, the invention provides the determination of tissue synthesis or breakdown rates of the molecule of interest. Such molecules of interest can be

indicative of a particular disease state or indicative of an inclination to develop a particular disease state. In another aspect, the invention provides the ability for diagnosis and medical management of a number of disease states or physiological states or conditions characterized by alterations in biological molecular synthesis and/or turnover rates, including, but not limited to, osteoporosis (*e.g.*, bone collagen synthesis and turnover rates); liver, cardiac, lung, and skin collagen synthesis rates in fibrogenic disorders (*e.g.*, hepatic cirrhosis, congestive heart failure, fibrotic lung disease, scleroderma and photo-aging); central nervous system amyloid precursor protein and amyloid fibril synthesis rate, proteolytic pathways, life-span and residence time in Alzheimer's disease; muscle myosin synthesis and turnover rates in wasting disorders, athletic training, and anabolic therapies; multiple sclerosis (brain myelination, demyelination and remyelination rates); rheumatoid arthritis and osteoarthritis (synovial fluid and articular cartilage synthesis and breakdown rates of joint protective glycosaminoglycans, and proteoglycans).

Other physiological states that can be diagnosed by the methods of the invention include, but are not limited to, osteoporosis, left-ventricular hypertrophy, liver cirrhosis, liver fibrosis, congestive heart failure, scleroderma, black-lung (coal-miner's pneumoconiosis), cardiac fibrosis, lung fibrosis, Alzheimer's disease, multiple sclerosis, rheumatoid arthritis, diabetes mellitus, muscle wasting syndromes, muscular dystrophies, athletic training, and cancer.

In another aspect, the invention is a method for monitoring a response of a disease or a condition in an individual to a therapeutic or disease-preventative intervention by assessing the rate of synthesis or breakdown of an biological molecule before the initiation of such intervention and then assessing the rate of synthesis or breakdown of the same biological molecule after the initiation of such therapeutic or disease-preventative intervention; and comparing both rates of synthesis or breakdown to monitor the response of a disease or a condition to the therapeutic intervention. In

one embodiment, the therapeutic intervention is an anabolic therapy. The rate of synthesis and breakdown of myosin in muscle is measured by the incorporation of a labeled precursor (*e.g.*,  $^2\text{H}_3$ -leucine,  $^2\text{H}_5$ -histidine,  $^{13}\text{C}$ -serine,  $^2\text{H}_2\text{O}$ ) into urinary 3-methylhistidine, both before anabolic therapy (*e.g.*, recombinant growth hormone, androgens, etc.) and after anabolic therapy. The rates after therapy are compared to the rates before therapy, to establish the effects of therapy.

In another aspect, the invention is a method for determining a risk for developing a disease state in an individual by determining the rate of synthesis or breakdown of an biological molecule indicative of the disease state and comparing the rate of synthesis or breakdown to a reference rate of synthesis or breakdown of the biological molecule wherein the reference rate reveals risk for the disease state. In one embodiment, the disease state is osteoporosis and the rate of synthesis and/or breakdown of bone collagen is the risk factor for developing this disease. The rate of synthesis and/or breakdown of bone collagen is measured based on the incorporation of a labeled precursor (*e.g.*,  $^{13}\text{C}_1$ -lysine,  $^2\text{H}_2\text{O}$ ) into a secreted metabolic derivative of bone collagen (*e.g.*, N-terminal telopeptides, deoxypyridinoline) in a subject and compared to reference values, to assess risk for the subsequent development of osteoporosis.

In another aspect, the invention is a method for determining a whole-body pool size of an biological molecule in an individual by: (1) measuring a daily fractional synthesis rate by the method using the method described above; (2) collecting the total excretion of an indicative metabolic derivative; (3) measuring the complete daily excretion rate (ER); and (4) dividing the daily ER by the daily fractional replacement rate of the metabolic derivative to calculate whole-body pool size of the biological molecule in the individual by use of the following equation:

$$\text{pool-size (g)} = \frac{\text{E.R. (g/d)}}{k (\text{d}^{-1})}$$

where  $k$  = fractional replacement rate constant. This can be used for assessing for the presence of cancer as exemplified in Example 5.

## V. Advantages provided by current invention

The invention has numerous advantages over previous techniques for measuring rates of biosynthesis and breakdown of biological polymers that require direct sampling of tissues by physical means. Most importantly, there is no requirement for direct tissue sampling, which in many circumstances is impractical, inconvenient, potentially risky, anxiety-provoking, or impossible in practice (*e.g.*, such as brain tissue sampling).

When comparing this invention to previous methods that measure the concentration or amount of a catabolic product released into the blood or urine, the present invention has a number of advantages. First, there is no requirement for quantitative recovery of metabolic derivatives. Previous techniques required complete or near-complete quantitative recovery of the metabolic derivatives from a biological sample in order to estimate the rate of production of the metabolic derivative (and from this, the rate of biosynthesis or breakdown of the molecule from which the metabolic derivative was derived). Factors that affect these techniques include *in vivo* clearance, storage or further metabolic transformations of the metabolic derivative. In contrast, the current invention requires only isolation of a quantity of the metabolic derivative that is sufficient for measurement of its isotopic labeling fraction, because the ratio of labeled to unlabeled metabolic derivatives or catabolic products is independent of the yield of metabolic derivatives or catabolic products attained. Accordingly, yield or recovery of the metabolic derivative is not a limiting factor or assumption of this invention and the method taught in the present invention can be performed rigorously regardless of variables such as *in vivo* clearance efficiency, metabolic transformation rate, storage, etc. of the metabolic derivative.

Second, in techniques in the prior art that involve measuring the concentration or amount of a metabolic derivative released into the bloodstream or urine, direct

estimation of only the breakdown rate of the biological molecule of interest was allowed (6), based on the rate of release of a metabolic derivative. In contrast, the present invention teaches a method for measuring aspects of biosynthesis (*e.g.*, synthesis rate, transit time, tissue residence time, etc.) in addition to breakdown rates of the molecule of interest.

**Table 2: Disease and Disorder, Tissues and Organs, and Associated Inaccessible Biological Molecule**

<b>Disease/Disorder</b>	<b>Tissue/Organ</b>	<b>"Inaccessible" Molecule</b>
Osteoporosis	Bone	Collagen
Photoaging (wrinkles)	Skin	Collagen
Liver fibrogenesis	Liver	Collagen
Cardiac fibrogenesis	Heart	Collagen
Pulmonary Fibrogenesis	Lung	Collagen
Scleroderma	Skin	Collagen
Arthritis (rheumatoid, osteo)	Joint	Glycosaminoglycans and proteoglycans
Alzheimer's Disease	Brain	Amyloid fibrils
DNA damage/mutation	Any tissue	Oxidized polynucleotides
Cell Proliferation Disorders, Cancer	Any tissue	Polynucleotides
Multiple Sclerosis	Brain	Myelin Basic Protein, Myelin membrane lipids (galactosyl-cerebrosides)
Frailty, wasting	Skeletal Muscle	Myosin
Brain development	Brain	Membrane lipids

**LITERATURE CITED:**

1. Wolfe, R. R. 1984. Tracers in Metabolic Research. Radio-Isotope and Stable Isotope/Mass Spectrometric Methods. Alan R. Liss, Inc., NY.
2. Hellerstein MK, Neese R. Mass isotopomer distribution analysis: a technique for measuring biosynthesis and turnover of polymers. *Am J Physiol* 263:E988-E1001, 1992.
3. Hellerstein MK, Neese RA. Mass isotopomer distribution analysis at eight years: theoretical, analytic and experimental considerations. *Am J Physiol* 276 (Endocrinol Metab 39): E1146-E1162, 1999.
4. Zilversmit, D. B., C. Entenman, and M. Fishler. 1943. The calculation of turnover rate and turnover time from experiments involving the use of labeling agents. *J. Gen. Physiol.* 26:325-331.

5. Hellerstein M. Methods for measuring polymerisation biosynthesis: three general solutions to the problem of the "true precursor." *Diabetes Nutr Metab* 13(1):46-60, 2000.
6. Waterlow, J. C., P. J. Garlick, and D. J. Millward, eds. 1978. *Protein Turnover in Mammalian Tissues and in the Whole Body*. North Holland, Amsterdam.
7. Ho DD, Neumann AU, Perelson AS, Chen W, Leonard JM, Markowitz M. Rapid turnover of plasma virions and CD<sub>4</sub> lymphocytes in HIV-1 infection. *Nature* 373:123-6, 1995.
8. Wei X, Ghosh SK, Taylor ME, Johnson VA, Emin ET, Deutsch P, Lifson JD, Bonhoeffer S, Nowak MA, Hahn BH et al. Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* 373 117-120, 1995
9. Schimke RT, Doyle D. Control of enzyme levels in animal tissues. *Annu Rev Biochem* 39: 929-76, 1970.
10. Eyre DR. Bone biomarkers as tools in osteoporosis management. *Spine* 22(24 Suppl): 17S-24S, 1997.
11. Young VR, Munro HN. Ntau-methylhistidine (3-methylhistidine) and muscle protein turnover: an overview. *Fed Proc* 37(9): 2291-300, 1978.

Example 1: Bone collagen biosynthesis using urinary pyridinoline/deoxypyridinoline (free or bone-collagen N-terminal peptide-derived) as the metabolic derivative

An individual with suspected or diagnosed osteoporosis or other disorder of bone collagen biosynthesis or breakdown is given a labeled precursor molecule that is incorporated into newly synthesized collagen in the body. In one such embodiment, this is <sup>13</sup>C<sub>1</sub>-lysine (at a dose of 20 mg/ml, in water, for example) given orally to drink (25 ml) with morning and evening meals for 7 days (total of 14 doses). A urine aliquot (10 ml) is collected from the individual at a defined time point or points (e.g., on the final day of the <sup>13</sup>C<sub>1</sub>-lysine protocol (day 7) and 7 days after completing the <sup>13</sup>C<sub>1</sub>-lysine intake protocol (day 14)).

In another embodiment, the individual is given labeled water in similar way to the  $^{13}\text{C}_1$ -lysine (for example, 50 ml of 70%  $^2\text{H}_2\text{O}$  twice a day for 7 days).

Urinary free or peptide-bound pyridinoline/deoxypyridinoline (HP/DP) are isolated (e.g., size exclusion chromatography or filtration to separate free from peptide-bound HP/DP; MW 1,000 cut-off; immunoprecipitation or immunoaffinity chromatography). Alternatively or in addition, to isolate specific N-terminal collagen peptides derived from bone, liver and heart are isolated (using antibodies available commercially), acid hydrolysis (6N HCl/110°C in sealed tube for 24 hr.); clean up of HP/DP by push-through SPE reversed-phase column; collection of HP/DP off reversed-phase C-18 HPLC column).

The HP/DP from a fraction of interest (e.g., total free or from a bone-collagen derived N-terminal peptide) that derives from bone collagen is injected into an LC/MS (e.g., BioQ electrospray/MS). Mass isotopomer peaks of HP ( $m/z$  429.2, 430.2, and 431.2) and DP (413.2, 414.2, 415.2) are monitored. Relative abundances of above mass isotopomers are quantified in the sample analyzed and in unlabeled standards (e.g.,  $m/z$  430.2/(429.2+430.2+431.2)=0.1650 in the day 14 sample and 0.1500 in the unlabeled standard). The proportion of labeled:unlabeled HP or DP molecules present is then calculated by MIDA methods.

By one such method, the proportion of excess labeled HP/DP molecules is calculated by subtraction of unlabeled standards from labeled samples (e.g., 0.0150, or 1.5%). Free lysine is isolated from the urine sample taken on the final day of  $^{13}\text{C}_1$ -lysine intake protocol (day 7) using an SPE column; then derivatized to butyl-ester acetamide-lysine, using butanolic HCl followed by acetic anhydride. The derivatized lysine is injected into a gas chromatograph/mass spectrometer (e.g., HP model 5973 instrument) using a DB-225 column. The mass spectrum of the lysine peak is collected, monitoring masses at  $m/z$  287 and 288 and quantifying their relative abundances compared to unlabeled standards (e.g.,  $m/z$  288/(287+288)=0.3752 in day 7 sample and 0.1433 in unlabeled standard).



The proportion of labeled:unlabeled free lysine molecules is calculated using mass isotopomer calculations, to establish the maximum proportion of label that could have been incorporated into tissue collagen during the  $^{13}\text{C}_1$ -lysine intake period (e.g., 30%). The ratio of the labeled proportion of HP or DP molecules derived predominantly from bone (total free HP/DP) or exclusively from bone (bone collagen N-terminal peptide), corrected for the 3 lysine labeling positions present, is compared to the labeled proportion of free lysine molecules and the ratio is calculated (e.g.,  $0.0050/0.3000$ , or 1.67%), based on the precursor-product equation; this ratio represents the fraction of bone collagen that was newly synthesized during the 7-day period of  $^{13}\text{C}_1$ -lysine intake.

The biosynthesis rate of bone collagen in the individual, during the period of  $^{13}\text{C}_1$ -lysine intake, is calculated (e.g., 0.24% per day, reflecting a doubling-time or replacement half-life of 289 days).

The above procedure and calculations are repeated in the same individual after a therapeutic intervention (e.g., treatment with conjugated estrogens, parathyroid hormone, calcium, bisphosphates, etc.), to determine the effects of the treatment on tissue collagen biosynthesis in the individual, or after a potential change in disease activity (e.g., bed-rest) to determine progression of osteoporosis in the individual.

Example 2: Tissue fibrogenesis using urinary pyridinoline/deoxypyridinoline derived from liver, heart, lung, or skin collagen N-terminal peptide as the metabolic derivative

An individual with a suspected or diagnosed fibrogenic disorder (e.g., hepatic fibrosis and/or cirrhosis, pulmonary interstitial fibrosis (PIF), or progressive cardiac failure) or with problematic skin photoaging (wrinkles) is given a labeled precursor that is incorporated into collagen during biosynthesis in the body. In one such embodiment, this is  $^2\text{H}_2\text{O}$  (at a dose of 50 ml, for example) given orally to drink with morning and evening meals for 42 days (6 weeks). A urine aliquot (10 ml) is

collected from the individual at a defined time point or points (e.g., on the final day of the  $^2\text{H}_2\text{O}$  protocol (day 42).

Urinary N-terminal collagen-peptides specific for liver, heart, or skin are isolated by, e.g., filtration to separate free HP/DP from collagen-derived peptides; immunoprecipitation or immunoaffinity chromatography to isolate specific N-terminal collagen-peptides released from liver or heart using antibodies available commercially; acid hydrolysis (6N HCl/110°C in sealed tube for 24 hr.); clean up of released HP/DP by push-through SPE reversed-phase column; collection of HP/DP off reversed-phase C-18 HPLC column. In one embodiment, the alanine and glycine released by hydrolysis from the peptide fraction of interest reflecting liver or heart collagen are derivatized (e.g. N-acetyl-butyl-ester of glycine or alanine, formed by reaction with butanolic HCl and acetic anhydride) is injected into a GC/MS according to conditions. Mass isotopomer peaks of glycine ( $m/z$  174, 175, and 176, representing parent,  $M_{+1}$  and  $M_{+2}$  ions) and alanine (188, 189, and 190 representing parent,  $M_{+1}$  and  $M_{+2}$  ions) are monitored.

Relative abundances of the above mass isotopomers are quantified in both the sample(s) and the unlabeled standards [e.g.,  $m/z$  189 / (188 + 189 + 190) = 0.1050 in the day 14 sample and 0.0900 in the unlabeled standard of alanine;  $m/z$  174 / (174 + 175 + 176) = 0.0935 for glycine in the day 14 sample and 0.0860 in the unlabeled standard]. The proportion of labeled:unlabeled alanine or glycine molecules present in the sample is then calculated.

By one such calculation method, the proportion of excess labeled:unlabeled alanine or glycine molecules present is calculated by subtraction of unlabeled standards from labeled samples (e.g., 0.0150, or 1.5% for alanine; 0.0075, or 0.75%, for glycine). The enrichment of body  $^2\text{H}_2\text{O}$  is measured (e.g. conversion to acetylene by addition to calcium carbide, then derivatization to the tetrabromo-ethane for analysis by gas chromatography/mass spectrometry). The proportion of labeled alanine or glycine present in tissue protein biosynthetic pools is then calculated,

based on body  $^2\text{H}_2\text{O}$  enrichments, using precursor-product equations and MIDA (e.g.  $0.1260 M_{+1}$ , or 3.1% excess  $M_{+1}$  for alanine if  $^2\text{H}_2\text{O} = 1.0\%$ ;  $0.1020 M_{+1}$  or 1.6% excess  $M_{+1}$  for glycine, if  $^2\text{H}_2\text{O} = 1.0\%$ ).

The labeled proportion of alanine or glycine molecules derived from liver or heart collagen is compared to the labeled proportion of alanine or glycine present in tissue protein biosynthetic pools and the ratio is calculated (e.g.,  $1.5\%/3.1\% = 48\%$  from alanine or  $0.75\%/1.6\% = 47\%$  from glycine), based on the precursor-product equation. This ratio represents the fraction of liver, heart, lung, or skin collagen that was newly synthesized during the 7-day period of  $^2\text{H}_2\text{O}$  intake. The biosynthesis rate of liver or heart collagen in the individual, during the period of  $^2\text{H}_2\text{O}$  intake, is then calculated (e.g., 1.6% per day, or a doubling-time or replacement half-life of 45 days).

The above procedure and calculations, or other calculation methods appropriate for precursor-product mathematical relationships are repeated in the same individual after a therapeutic intervention (e.g., treatment with antifibrogenic agents) to determine the effects of the treatment on liver or cardiac fibrogenesis in the individual, or after a potential change in disease activity (e.g., cessation of alcohol intake in an individual with liver fibrosis, treatment of with antifibrigenic or anti-inflammatory agents in PIF, treatment of cardiac failure with angiotensin-converting enzyme inhibitors in an individual with cardiac fibrosis) to determine the progression of underlying fibrogenesis in the individual.

### Example 3: Brain Amyloid Precursor Protein (APP) biosynthesis using amyloid (A)-beta 1-40 and 1-42 peptides as the metabolic derivatives

An individual concerned about risk for Alzheimer's disease or who has been diagnosed with early Alzheimer's disease is given a labeled precursor that is incorporated into newly synthesized proteins in the body. A 10% solution of  $^{13}\text{C}_1$ -glycine is given orally (100 mg/ml, in water) every 2 hours for a total of 3 doses (time

zero, two and four hours; 30 ml doses, for total of 90 ml). A plasma (2 ml) sample is collected from the individual after a known time (*e.g.*, 6 hours) of  $^{13}\text{C}_1$ -glycine intake. A urine aliquot (20 ml) is collected from the individual at a defined time point or points (*e.g.*, day 3 after administration of the  $^{13}\text{C}_1$ -glycine). From the urine aliquot, total amyloid-beta (A-beta) peptides are immunoprecipitated, for example, using a monoclonal anti-A-beta antibody coupled to Sepharose beads (Senetek, Inc.), then eluted from the beads with isopropanol/water formic acid (4:4:1), plus cyano-4-hydroxycinnamic acid. The A-beta peptides are loaded onto matrix-assisted laser desorption (MALDI)/time-of-flight (TOF) mass spectrometer (1.5 microliter added). Mass isotopomers in the A-beta 1-40 envelope (*e.g.*,  $m/z$  4,327-4,335) and A-beta 1-42 (*e.g.*,  $m/z$  4,511-4,519) are monitored.

Relative abundances of the above mass isotopomers are quantified in the sample and compared to values from unlabeled standards to calculate the proportion of labeled molecules present in the sample.

By one such calculation method, the proportion of excess labeled A-beta molecules is calculated by subtraction of unlabeled standards from labeled samples (*e.g.*, sum of  $m/z$  4,327-4,335=0.0040 above the value in standards of A-beta 1-40 and the um of  $m/z$  4.511-4.519 is 0.0030 above this value in standards of A-beta 1-42). Free glycine is isolated from the plasma sample using an SPE column and converted to the butyl ester-acetamide derivative, *e.g.* by using butanolic HCl followed by acetic anhydride. The derivatized glycine is injected into a gas chromatograph/mass spectrometer (*e.g.*, HP Model 5973) using a DB-225 column and conditions. The mass spectrum of the glycine peak is collected, monitoring masses  $m/z$  174 and 175, and quantifying their relative abundances compared to unlabeled standards (*e.g.*,  $m/z$  175/(174+175)=0.4349 in sample and 0.0872 in standard). The proportion of labeled:unlabeled glycine molecules present is calculated, to establish the maximal possible label incorporation into brain APP during the period of  $^{13}\text{C}_1$ -glycine intake (*e.g.*, 40%).

The ratio of the labeled proportion of A-beta 1-40 or A-beta 1-42 molecules is then compared to the labeled proportions of free glycine molecules, by one calculation method, correcting for the number of glycine subunits in A-beta 1-40 or A-beta 1-42; these ratios represent the fraction of brain APP converted to A-beta 1-40 and A-beta 1-42 that were newly synthesized during the  $^{13}\text{C}_1$ -glycine labeling period; any differences between A-beta 1-40 and A-beta 1-42 represent differences in biosynthesis of brain APP destined for A-beta 1-40 and A-beta 1-42, respectively. The biosynthesis rate of APP in the brain of the individual during the period of  $^{13}\text{C}_1$ -glycine administration is calculated by MIDA, (*e.g.*, 1.0% biosynthesis/6 hour, or 4.0% per day, or a residence time of 25 days ( $1.0/0.04$ ) for brain APP in the individual).

The same procedure is repeated in the individual after an experimental intervention (*e.g.*, secretase inhibitor; estrogen treatment), to determine the effects of the treatment on brain total APP biosynthesis and turnover, as one example, or on partitioning between A-beta 1-40 and A 1-42, as another example, as an index of efficacy of the intervention, or after a potential change in disease activity (*e.g.*, subjective change in mental status) as an index of progression of underlying Alzheimer's risk or disease activity.

Alternatively, said individual is given  $^2\text{H}_2\text{O}$  (70%, 50 ml twice a day by mouth) for 14 days. A plasma, saliva or urine sample is taken from the subject at days 7 and 14 of  $^2\text{H}_2\text{O}$  intake. A urine aliquot is collected from the individual at day 14. From the urine aliquot, total A-beta peptides or A-beta 1-40 and A-beta 1-42 peptides are isolated by immunoprecipitation, as described above. The A-beta peptides are then subjected to acid hydrolysis ( $\text{HCl}$   $100^\circ\text{C}$ , 60 min.) to release free amino acids. The amino acids are derivatized for GC/MS analysis.

By one such method, the N-acetyl-butyl-ester derivative of alanine is formed, using butanolic  $\text{HCl}$  followed by acetic anhydride.

The derivatized amino acids are injected into a GC/MS (e.g. HP 5973 instrument) using a DB-225 column. The mass spectrum of the alanine and glycine peaks are collected. Relative abundances of  $m/z$  188-190 are measured in alanine in samples and unlabeled standards (e.g. alanine  $m/z$  188/( $m/z$  188+189+190) = 0.1025 in samples and 0.0950 in standards). The proportion of labeled:unlabeled alanine molecules present in the sample is then calculated.

By one such method, the proportion of excess labeled alanine molecules in the sample is calculated by subtraction of the relative abundance of  $M_{+1}$  alanine in unlabeled standards from labeled samples (e.g.  $0.1025 - 0.0950 = 0.0075$ , or 0.75% labeled  $M_{+1}$  alanine molecules).

These relative abundances are compared to calculated maximal possible abundances at the measured body  $^2\text{H}_2\text{O}$  enrichment present (e.g. in body  $^2\text{H}_2\text{O}$  enrichment = 1.0%, alanine excess  $M_{+1}$  abundance = 3.1%). The fractional synthesis rate of the A-beta peptide isolated is calculated using standard precursor-product equations known in the art (e.g. measured alanine excess  $M_{+1} = 0.75\%$ , calculated maximal alanine excess  $M_{+1} = 3.1\%$ , ratio =  $0.75/3.1\% = 24\%$  new A-beta synthesis over 14 days, or 1.96% replacement of brain A-beta from APP per day).

The above procedure is repeated in the individual after an experimental intervention, for example, as described above.

Example 4: Synovial fluid and cartilage hyaluronan or other glycosaminoglycans or proteoglycan biosynthesis and breakdown rates using hyaluronic disaccharide polymers and chondroitin-sulfate polymers as the metabolic derivative

An individual with established or suspected rheumatoid arthritis (RA) or osteoarthritis (OA) or at risk for RA or OA is given a labeled precursor that is incorporated into a glycosaminoglycan or proteoglycan in the synovial joint fluid or cartilage (such as hyaluronan, chondroitin sulfate, heparan-sulfate or others). Examples of labeled precursors include  $^2\text{H}_2\text{O}$  (incorporated into the N-acetyl-

glucosamine and glucuronic acid moieties of hyaluronan; into the N-acetyl-galactosamine-sulfate and glucuronic acid moieties of chondroitin-sulfate, or the N-acetyl-glucosamine-sulfate and glucuronic acid-sulfate moieties of heparan-sulfate);  $^2\text{H}$ - or  $^{13}\text{C}$ -glucose (incorporated into the N-acetyl-galactosamine and N-acetyl-glucosamine moieties of these glycosaminoglycans and proteoglycans);  $^{13}\text{C}$ -acetate (incorporated into the acetyl-moieties of N-acetyl-galactosamine or N-acetyl-glucosamine); and  $^{15}\text{N}$ -glycine (incorporated into the nitrogen component of N-acetyl-galactosamine or N-acetyl-glucosamine). A blood or urine aliquot is collected from the individual. High performance-liquid-chromatography (HPLC) is performed to isolate polymers of hyaluronic acid-disaccharide ( $\text{HA}_n$ ), polymers of chondroitin-sulfate-disaccharide ( $\text{CS}_n$ ) and/or polymers of other glycosaminoglycan disaccharides, such as the polymer of heparan-sulfate-disaccharide ( $\text{HS}_n$ ), using HPLC procedures understood in the art. Alternatively, the polymers of these glycosaminoglycan-disaccharides can be converted to their free disaccharide units (e.g. by incubation of the sample with hyaluronidase). The glycosaminoglycan disaccharide polymers or free disaccharide units are then derivatized, to allow one of its components to be analyzed by GC/MS (by treating with methanolic HCL [ $100^\circ\text{C}$ ] followed by acetic anhydride:pyridine, to produce the methyl, triacetyl, N-acetylglucosamine derivative from  $\text{HA}_n$  or HA, for example).

The isotopic enrichment of said derivatized component is then measured by GC/MS, such as selected ion monitoring of the appropriate masses. For example, the methyl, triacetyl, N-acetylglucosamine derivative is analyzed as  $m/z$  331-333 (representing the  $M_0$ ,  $M_{+1}$  and  $M_{+2}$  masses). Relative abundances of the above mass isotopomers are quantified in labeled samples and compared to unlabeled standards (e.g.  $332/(331+332+333)=0.1370$  in unlabeled standards). An example of one such mass spectrum is shown in Figure 9.

The proportion of labeled to unlabeled molecules present in each sample is then calculated. By one such calculation method, the proportion of excess labeled methyl-

triacetyl-N-acetyl-glucosamine molecules in the sample is calculated by subtracting unlabeled standards from labeled samples (e.g. 0.1540  $M_{+1}$  in samples, 0.1370  $M_{+1}$  in standards, or 0.0170  $M_{+1}$  = 1.70% labeled  $M_{+1}$  methyl-triacetyl-N-acetyl-glucosamine). The proportion of labeled N-acetyl glucosamine molecules present in tissue hyaluronan biosynthetic pool is calculated based on body  $^2\text{H}_2\text{O}$  enrichments, using MIDA (e.g. 0.1710  $M_{+1}$  N-acetyl-glucosamine in tissue pools if body  $^2\text{H}_2\text{O}$  enrichment is 1.0%, or 3.4% labeled  $M_{+1}$  N-acetyl-glucosamine). The biosynthesis and breakdown rates of synovial fluid or hyaline cartilage hyaluronan are then calculated by comparison of the proportion of labeled N-acetyl-glucosamine molecules in the sample to the proportion of labeled molecules in tissue N-acetylglucosamine pools (e.g. 1.70%/3.4% = 50% newly synthesized HA molecules in synovial fluid and cartilage).

The rate of hyaluronan or other glycosaminoglycan biosynthesis and breakdown in synovial fluid and cartilage reflects the replacement and destruction rates of joint glycosaminoglycans and can be used as a measure of disease activity and/or therapeutic efficacy in RA or OA, particularly for assessment of joint protective anti-rheumatic agents.

The above procedure and calculations may be repeated after an intervention intended to stimulate production of hyaluronan, chondroitin-sulfate or other synovial glycosaminoglycans (such as glucosamine-sulfate) and to slow the progression of RA or OA, as an index of efficacy of the intervention, or after an apparent change in disease activity (e.g. a new set of symptoms), as an index of or test for disease activity or progression.

#### Example 5: Muscle Myosin biosynthesis using urinary 3-Methyl-Histidine as the metabolic derivative

An individual undergoing a physical training program or medical therapeutic regimen intended to increase muscle mass by increasing muscle myosin biosynthesis



(e.g., an athlete; an elderly person receiving physical therapy after a stroke; a patient with cachexia related to cancer or AIDS who is receiving nutritional or anabolic agent therapy) is given a labeled precursor that is incorporated into the body's proteins during biosynthesis. Examples of labeled precursors include  $^{15}\text{N}$ -histidine (50 mg/ml in water), given orally every 2 hours for 4 doses (10 ml/dose). A urine aliquot (10 ml) is collected from the individual at a defined time point or points (e.g., at the conclusion of the  $^{15}\text{N}$ -histidine or 3- $^{13}\text{C}$  serine administration protocol and again at day 3 after administration of the  $^{15}\text{N}$ -histidine. In an alternative embodiment,  $^2\text{H}_2\text{O}$  is given (50 ml of 70%  $^2\text{H}_2\text{O}$ ) twice a day for 7 days. Total urinary amino acids are isolated with an SPE column. The amino acids are derivatized for gas chromatographic/mass spectrometric analysis.

By one such method, the amino acids are converted to the butyl-ester acetamide derivative, using butanolic HCl followed by acetic anhydride.

The derivatized amino acids are injected into a gas chromatograph/mass spectrometer (e.g., HP 5973 instrument), using, e.g., a DB-225 column. The mass spectrum of the 3-methyl histidine peak is collected while monitoring mass isotopomers at  $m/z$  267 and 268 (parent and  $M+1$  ions, respectively). Relative abundances of the above mass isotopomers are quantified in samples and compared to unlabeled standards (e.g.,  $m/z$  267/[ $m/z$  267+268] = 0.1200 in samples and 0.0960 in standards). The proportion of labeled:unlabeled molecules present in the sample is calculated for 3-methylhistidine using MIDA.

By one such calculation method, the proportion of excess labeled 3-methyl-histidine molecules in the sample is calculated by subtraction of unlabeled standards from labeled samples (e.g., 0.0240, or 2.4%). The mass spectrum of the derivatized-histidine or serine peaks (from the timepoint collected at the conclusion of the isotope administration protocol) are collected while monitoring mass isotopomers at 263 and 264 (parent and  $M+1$  ions, respectively) for histidine or mass isotopomers at 246 and 247 (parent and  $M+1$  ions, respectively) for serine. The relative abundances of the

above mass isotopomers in samples are quantified and compared to unlabeled standards (e.g.,  $m/z$  264/(263+264)=0.4450 in samples and 0.0950 in standards, for histidine). The ratio of labeled to unlabeled molecules is calculated for histidine or serine (e.g., 0.3500, or 35%).

From the proportion of labeled 3-methylhistidine molecules present compared to the proportion of labeled free histidine or free serine molecules present, the biosynthesis rate of new muscle myosin in the individual during the 8 hour period of  $^2\text{H}_5$ -histidine administration is calculated, using standard precursor-product equations (e.g.,  $0.0240/0.3500=6.8\%$ ).

The above procedure is repeated periodically, to establish the efficacy of the individual's athletic training program or medical therapeutic program intended to increase muscle myosin biosynthesis.

An example of this procedure in human subjects is shown in Figure 8, which demonstrates that metabolic derivative in blood may be used to reflect label incorporation in molecule in brain. Table 3 shows the incorporation of  $^2\text{H}$  from  $^2\text{H}_2\text{O}$  into urinary 3-methyl-histidine in human subjects during intake of  $^2\text{H}_2\text{O}$  for 4-8 weeks. The data suggest a half-life of roughly 3 weeks for muscle myosin in these human subjects.

**Table 3: Incorporation of  $^2\text{H}$  from  $^2\text{H}_2\text{O}$  into urinary 3-methyl-histidine in human subjects during intake of  $^2\text{H}_2\text{O}$  for 4-8 weeks.**

EM1: excess M+1 mass isotopomer in t-butyl dimethyl silyl derivative of 3-methyl histidine;  $^2\text{H}_2\text{O}$  (%): body water enrichment;  $A_1^\infty$ : calculated maximal EM1 of 3-methyl histidine at measured  $^2\text{H}_2\text{O}$ ; f(%): fractional synthesis; k: replacement rate constant of 3-methyl-histidine.

EM1	$^2\text{H}_2\text{O}$ (%)	$A_1^\infty$	f (%)	k ( $\text{d}^{-1}$ )
2.6	1.8	3.3	78	0.037
$\pm 1.5$	$\pm 0.3$	$\pm 0.5$		

Example 6: Whole-body cell division (DNA biosynthesis) using methyldeoxycytosine as the metabolic derivative

A human individual at risk for cancer or other disorder related to cell proliferation is given a labeled precursor that is incorporated into newly synthesized DNA in the body.  $^2\text{H}_2\text{O}$  is given orally (70%  $^2\text{H}_2\text{O}$  as drinking water, 80 ml once a day for 14 days). A urine aliquot (< 10 ml) is collected from the individual at a defined time point (e.g., at the completion of the 14-day  $^2\text{H}_2\text{O}$  administration period). Total urinary nucleosides are isolated with an SPE column, then the fractions enriched with deoxycytosine and methyl-deoxycytosine are eluted with a water wash. The isolated nucleosides including methyldeoxycytosine are derivatized for gas chromatographic/mass spectrometric analysis, according to methods known calculation methods. By one such method, the trimethylsilyl (TMS) derivative of methyldeoxycytosine and other nucleosides present is formed with bis(trimethyl[silyl])acetamide. The TMS methyl-deoxycytosine is injected into a gas chromatograph/mass spectrometer (e.g., HP model 5973), with a DB-17 column.

The mass spectrum of the methyldeoxycytosine peak is collected monitoring mass isotopomers at  $m/z$  457-459 (parent, M+1 and M+2 ions). Relative abundances of the above mass isotopomers are quantified in samples and compared to unlabeled standards (e.g., 0.0950 M+1 in samples, 0.0820 M+1 in unlabeled standards). The proportion of labeled:unlabeled molecules present in the sample is calculated for methyldeoxycytosine.

By one such calculation method, the proportion of excess labeled methyldeoxycytosine molecules in the sample is calculated by subtraction of unlabeled standards from labeled samples (e.g., 0.0130 or 1.3% labeled methyldeoxycytosine).

From the proportion of labeled methyldeoxycytosine molecules present, the biosynthesis of total body DNA during the 14 day  $^2\text{H}_2\text{O}$  labeling period is calculated using standard precursor-product equations and estimates or direct measurements of the maximal deoxycytosine enrichment at these  $^2\text{H}_2\text{O}$  administration rates (e.g., 15% new DNA/14 days, or biosynthesis rate of ca. 1.1% per day).

The above procedure is repeated after an intervention intended to reduce cell proliferation (DNA biosynthesis) throughout the body, such as caloric restriction, vitamin D administration, or cell-cycle inhibitory drugs, and thereby reduce general cancer risk in the individual.

Example 7: Brain membrane lipid biosynthesis (brain growth and development) using 24(S)-hydroxycholesterol in plasma as the metabolic derivative

A human individual is given a labeled precursor that is incorporated into newly synthesized lipids in the body.  $^2\text{H}_2\text{O}$  is administered orally for a defined period of time (e.g., 70%  $^2\text{H}_2\text{O}$  as drinking water, 80 ml once a day, for 7 days). A blood aliquot is collected from the individual at a defined time point. 24(S)-hydroxycholesterol, a catabolite of tissue cholesterol that is uniquely synthesized from cholesterol in brain and escapes into the bloodstream, is extracted from blood, and derivatized for gas chromatographic/mass spectrometric measurement.

The isotopic enrichment of  $^2\text{H}$ -24(S)-hydroxycholesterol is determined by MIDA, from the ion abundances at m/z 458, 459 and 460 in samples compared to unlabeled standards (e.g., 0.1000 in sample, 0.0900 in unlabeled standards). The proportion of labeled to unlabeled molecules of 24(S)-hydroxycholesterol present in the sample is calculated.

By one such calculation method, the proportion of excess labeled 24(S)-hydroxycholesterol molecules in the sample is calculated by subtraction of unlabeled standards from labeled samples (e.g., 0.0100 or 1%). The isotopic enrichment of body water is determined by MIDA (e.g., 1.5%).

The biosynthesis rate of brain cholesterol, and thus brain myelin (ratio of 2:1, cholesterol:ceramide, in myelin), is determined by application of the precursor-product relationship from labeled hydrogen in body water to labeled hydrogen in newly synthesized cholesterol (e.g., 2.1% new 24(S)-hydroxycholesterol over 7 days, or biosynthesis rate of brain cell membranes of 0.3% per day, for a doubling-time or half-life of 231 days).

The above procedure is repeated after an intervention intended to stimulate brain growth and/or development (e.g., a dietary intervention, educational program or other stimulatory activity in children, pharmacologic therapy), to establish efficacy of the intervention.

Example 8: Brain myelin biosynthesis and breakdown rates (myelination, demyelination and remyelination) using plasma galactosyl-cerebroside as the metabolic derivative

A human individual with a known or suspected demyelinating disorder, such as multiple sclerosis, is given a labeled precursor that is incorporated into a lipid moiety that is exclusively or nearly exclusively present in the brain myelin sheath (such as galactosyl-cerebroside) and that is released into the bloodstream or cerebrospinal fluid after breakdown of brain myelin. Such labeled precursors include  $^2\text{H}_2\text{O}$  (incorporated into the galactose, sphingosine and fatty acid moieties of galactosyl-cerebrosides in the myelin sheath),  $^2\text{H}$ -glucose or  $^{13}\text{C}$ -glucose (incorporated into the galactose moiety of galactosyl-cerebroside),  $^{13}\text{C}$ -serine (incorporated into the sphingosine moiety of cerebroside) or  $^{13}\text{C}$ -fatty acids (incorporated into the fatty acyl-moiety of galactosyl-cerebrosides). A blood or urine aliquot is collected from the individual. Lipids are extracted from the blood or urine sample, for example, by Folch extraction. Galactosyl-cerebroside- or another characteristic lipid components of the myelin sheath is then separated from the lipid extract, such as thin layer chromatography. The galactosyl-cerebroside is then derivatized to allow one of its

components to be analyzed by gas chromatography/mass spectrometry (GC/MS), such as methanolic HCl followed by acetic anhydride-pyridine to produce methyl, triacetyl-galactose, or methanolic HCl to produce fatty acid-methyl ester, to produce derivatives of sphingosine.

The isotopic enrichment of the labeled component of galactosyl-cerebroside analyzed (e.g. the derivatized galactose, fatty acid or sphingosine) is then measured by selected ion monitoring of the appropriate masses. In the example of an individual given  $^2\text{H}_2\text{O}$  for 4 weeks and where the galactose moiety of galactocerebroside from plasma is analyzed, as  $m/z$  331, 332 and 333 of methyl-tetracetyl-galactose, representing parent,  $M_{+1}$  and  $M_{+2}$  ions, is measured by selected ion monitoring during GC/MS analysis of samples and unlabeled standards. Relative abundances of the above mass isotopomers are quantified in samples and compared to unlabeled standards (e.g. 0.1450  $M_{+1}$  in samples, 0.1350  $M_{+1}$  in unlabeled standards). The proportion of labeled to unlabeled molecules present in each sample is then calculated. By one such calculation method, the proportion of excess labeled methyl, triacetyl-galactose molecules in the sample is calculated by subtraction of unlabeled standards from labeled samples (e.g. 0.0100 or 1.00% labeled  $M_{+1}$  methyl-tetracetyl-galactose in the above example). The proportion of labeled galactose molecules present in tissue galactosyl-cerebroside biosynthetic pools is calculated based on body  $^2\text{H}_2\text{O}$  enrichments, using MIDA (e.g. 0.1680  $M_{+1}$  in tissue pools if body water  $^2\text{H}_2\text{O}$  enrichment is 1.0%, or 3.3% labeled  $M_{+1}$ ). The biosynthesis and breakdown rates of brain myelin-sheath lipids are then calculated by comparison of the proportion of labeled galactose molecules in the sample to the proportion of labeled galactose molecules in tissue galactosyl-cerebroside biosynthetic pools (e.g.  $1.00\%/3.3\% = 30\%$  newly synthesized galactosyl-cerebroside in brain myelin over the period of  $^2\text{H}_2\text{O}$  intake by the subject). The rate of galactosyl-cerebroside biosynthesis and breakdown reflects the rate of myelination, demyelination and

remyelination in brain and may be used as a measure of disease activity and/or therapeutic efficacy in multiple sclerosis or other clinical demyelinating conditions.

The above procedure and calculations may be repeated after an intervention intended to stimulate remyelination or reduce demyelination and slow the progression of multiple sclerosis, as an index of efficacy of the intervention, or after an apparent change in disease activity (e.g. a set of new symptoms), as an index of or test for disease activity or progression.

An example of this procedure is shown in Figure 9, which demonstrates measurement of isotopic enrichment in HA through GC/MS procedures.

Example 9: Brain myelin biosynthesis and breakdown (myelination, demyelination, and remyelination), from myelin basic protein like material (MBPLM) in urine as the metabolic derivative

A human individual with a known or suspected demyelinating disorder, such as multiple sclerosis, is given a labeled precursor that is incorporated into newly synthesized proteins in the body (e.g.  $^2\text{H}_2\text{O}$  or a labeled amino acid such as  $^{13}\text{C}$ -leucine) for a defined period of time, such as 4 weeks. A blood aliquot is collected from the individual. Myelin basic protein like material (MBPLM) is isolated from blood by use of a specific antibody, for example, by using an immunoaffinity column. The MBPLM is hydrolyzed to free amino acids, using acid conditions or protease enzymes. The free amino acids are derivatized for analysis by gas chromatography/mass spectrometry.

The isotopic enrichment of the labeled amino acid(s) isolated from MBPLM is measured by selected ion monitoring on the appropriate masses (e.g.,  $m/z$  231, 232 and 233 for n-butyl-ester-acetamide of leucine, if  $^{13}\text{C}_1$ -leucine was administered to the subject or  $m/z$  188-190 for the N-acetyl-butyl ester derivative of alanine, if  $^2\text{H}_2\text{O}$  was administered to the subject) in samples and unlabeled standards.

The proportion of labeled to unlabeled leucine or alanine molecules present in the sample is calculated.

By one such calculation method, the proportion of excess labeled alanine molecules in the sample is calculated by subtraction of the relative abundance of  $M_{+1}$  alanine in unlabeled standards from labeled samples (e.g.  $M_{+1}$  alanine in unlabeled standards is 0.0950,  $M_{+1}$  alanine in labeled samples is 0.1050, so the proportion of labeled alanine molecules in the sample is 0.0100 or 1.0%). The proportion of labeled alanine present in tissue protein biosynthetic pools is then established, based on the  $^2\text{H}_2\text{O}$  enrichment of body water (e.g. 3.1%  $M_{+1}$  alanine, or 0.1260  $M_{+1}$  alanine in tissue pools, if body water  $^2\text{H}_2\text{O}$  enrichment is 1.0%). The biosynthesis and breakdown rates of brain MBPLM are then determined by comparison of the proportion of labeled alanine molecules present in the MBPLM to the proportion of labeled alanine present in tissue protein biosynthetic pools (e.g.  $1.0\%/3.1\% = 33\%$  biosynthesis of MBPLM over 4 weeks), by application of the precursor-product relationship or other equations known in the art.

The rate of MBPLM biosynthesis and/or breakdown reflects the rate of myelination, demyelination and remyelination and may be used as a measure of disease activity, and/or therapeutic efficacy, in multiple sclerosis or other clinical demyelinating conditions.

The above procedure and calculations may be repeated after an intervention intended to stimulate remyelination or reduce demyelination and slow the progression of multiple sclerosis, as an index of efficacy of the intervention, or after a potential change in disease activity (e.g., new symptoms of uncertain cause), as an index of or test for disease activity or progression.

Example 10: Tissue collagen biosynthesis from the rate of dilution of label in pyridinoline/ deoxypyridinoline after discontinuing label administration



The same procedure as described above (see examples 1 or 2), for tissue collagen biosynthesis using pyridinoline/deoxypyridinoline (HP/DP), is followed (through calculation of the proportion of labeled:unlabeled HP and DP molecules present, at a defined time (*e.g.*, time zero) after administration of  $^{13}\text{C}_1$ -lysine or other labeled precursor for tissue collagen biosynthesis). A urinary aliquot or a plurality of urinary aliquots (10 ml) are collected subsequently, at a defined time-point or points after time zero (*e.g.*, every 2 weeks for 2 months in one embodiment).

The same analytic procedure is followed as for the original urinary sample (see examples 1 or 2), to calculate the proportion of labeled:unlabeled HP and DP molecules present at each time point. From the dilution rate (*i.e.*, the rate of decrease in the proportion of labeled DP/HP molecules present), the biosynthesis rate ( $k$ ) of tissue collagen is calculated, using the standard isotope dilution equation using the formula:

$$A_t = A_0 \cdot e^{-kt},$$

where  $A_t$  = proportion of labeled DP/HP in sample at time  $t$

$A_0$  = proportion of labeled DP/HP in sample at time zero

$t$  = time

$k$  = rate constant for tissue collagen biosynthesis

$$k = \frac{-\ln\left(\frac{A_t}{A_0}\right)}{t},$$

The same procedure is repeated in the individual after a therapeutic intervention intended to alter the biosynthesis rate of bone collagen or other tissue collagens is performed, as an index of efficacy of the intervention.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

## CLAIMS

What is claimed is:

1. A method for determining the rate of biosynthesis or breakdown of one or more inaccessible biological molecules in an individual comprising the steps of:
  - a) administering an isotope-labeled precursor molecule to an individual for a period of time sufficient for the label of said isotope-labeled precursor molecule to become incorporated into said one or more inaccessible biological molecules;
  - b) obtaining one or more accessible biological samples from an individual, wherein said one or more accessible biological samples comprise one or more metabolic derivatives of said one or more inaccessible biological molecules as resulting from *in vivo* metabolism of said inaccessible biological molecules; and
  - c) detecting the incorporation of said label in said one or more metabolic derivatives by mass spectrometry to determine said rate of biosynthesis or breakdown of said one or more inaccessible biological molecules.
2. The method according to claim 1 wherein said detecting step includes calculating the isotope enrichment of said inaccessible biological molecule by mass isotopomer distribution analysis (MIDA); and applying precursor-product or exponential decay equations to determine the rate of biosynthesis or breakdown of said inaccessible biological molecule.
3. The method according to claim 1 wherein said one or more precursor molecules are administered *in vivo*.
4. The method according to claim 1 wherein the isotopic label is selected from the group consisting of  $^2\text{H}$ ,  $^3\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^{18}\text{O}$ ,  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{35}\text{S}$ ,  $^{32}\text{P}$ ,  $^{125}\text{I}$ , and  $^{131}\text{I}$ .
5. The method of claim 4 wherein said label is  $^2\text{H}$ .

6. The method of claim 1 wherein said precursor molecule is water.
7. The method according to claim 1 comprising the additional step of partially purifying said one or more metabolic derivatives from said accessible biological samples before said step (c).
8. The method according to claim 1 wherein said isotope-labeled precursor molecule is administered orally.
9. The method according to claim 1 wherein following said step (b), the method includes the additional step of degrading said one or more metabolic derivatives to form degraded metabolic derivatives.
10. The method according to claim 9, wherein said degraded metabolic derivatives are further separated by gas chromatography or HPLC.
11. The method according to claim 1 wherein said individual is a human.
12. The method of claim 1, wherein said metabolic derivatives are catabolic products.
13. The method of claim 1, wherein said metabolic derivative derives primarily from said one or more inaccessible biological molecules.
14. The method according to claim 1 wherein said label of said isotope-labeled precursor molecule is incorporated into said one or more inaccessible

biological molecules followed by catabolic breakdown of said one or more inaccessible biological molecules to form said one or more metabolic derivatives.

15. The method according to claim 1 wherein said one or more metabolic derivatives cannot be utilized in the biosynthesis of another inaccessible biological molecule in said individual.

16. The method according to claim 1 wherein said biosynthesis or breakdown does not occur in said one or more accessible biological samples of step (b).

17. The method according to claim 1 comprising the additional step of discontinuing said administering step (a).

18. The method according to claim 1 wherein said one or more inaccessible biological molecules are selected from the group consisting of proteins, polynucleotides, lipids, glycosaminoglycans, prostoglycans, and carbohydrates.

19. The method according to claim 18 wherein said one or more inaccessible biological molecules is a protein.

20. The method according to claim 19 wherein said precursor molecule is an amino acid or one or more metabolic precursors of an amino acid.

21. The method according to claim 19 wherein said label is incorporated post-translationally into said protein.

22. The method according to claim 19 wherein said one or more metabolic derivatives is an amino acid or peptide.

23. The method according to claim 19 wherein said protein is collagen.

24. The method according to claim 23 wherein said one or more metabolic derivatives include a collagen-specific metabolic derivative selected from the group consisting of pyridinoline, deoxypyridinoline, hydroxyproline, hydroxylysine, glucosylgalactosyl-hydroxylysine, galactosylhydroxylysine, N-terminal telopeptide  $\alpha$ (I) (SEQ ID NO:1), N-terminal telopeptide  $\alpha_2$ (I) (SEQ ID NO:2), N-terminal telopeptide  $\alpha$ 2(I) (SEQ ID NO:3), N-terminal telopeptide  $\alpha$ 1(II) (SEQ ID NO:4), N-terminal telopeptide  $\alpha$ 1(III) (SEQ ID NO:5), C-terminal telopeptide  $\alpha$ 1(I) (SEQ ID NO:6), C-terminal telopeptide  $\alpha$ 2(I) (SEQ ID NO:7), C-terminal telopeptide  $\alpha$ 1(II) (SEQ ID NO:8), C-terminal telopeptide  $\alpha$ 1(II) (SEQ ID NO:9), C-terminal telopeptide  $\alpha$ 1(II) (SEQ ID NO:10), C-terminal telopeptide  $\alpha$ 1(III) (SEQ ID NO:11), cross-linked carboxy-terminal peptide of type I collagen (ICTP), PINP( $\alpha$ 1) (SEQ ID NO:12), PICP( $\alpha$ 1) (SEQ ID NO:13), PINP( $\alpha$ 2) (SEQ ID NO:14), PICP( $\alpha$ 2) (SEQ ID NO:15), PIINP( $\alpha$ 1) (SEQ ID NO:16), PIICP( $\alpha$ 1) (SEQ ID NO:17), PIIINP( $\alpha$ 1) (SEQ ID NO:18), PIIICP( $\alpha$ 1)(SEQ ID NO:19), PIVNP( $\alpha$ 1)(SEQ ID NO:20), PIVNP( $\alpha$ 2)(SEQ ID NO:21), PIVNP( $\alpha$ 2)(SEQ ID NO:22), PIVNP( $\alpha$ 3) (SEQ ID NO:23), PIVNP( $\alpha$ 4) (SEQ ID NO:24), PIVNP( $\alpha$ 5) (SEQ ID NO:25), and PIVNP( $\alpha$ 6) (SEQ ID NO:26).

25. The method of claim 24 wherein said one or more metabolic derivatives are an N-terminal or C-terminal amino acid sequence specific to a type of collagen.

26. The method according to claim 19 wherein said protein is myosin and said metabolic derivative is 3-methylhistidine.

27. The method according to claim 19 wherein the said protein is Amyloid Precursor Protein (APP) and said metabolic derivative is an APP-specific metabolic derivative.

28. The method according to claim 27 wherein said APP-specific metabolic derivative is amyloid-beta 1-40 or amyloid-beta 1-42.

29. The method according to claim 19 wherein said one or more metabolic derivatives is a post-translationally modified amino acid or protein.

30. The method according to claim 29 wherein said post-translationally modified amino acid or protein is selected from the group consisting of phosphorylated, methylated, hydroxylated, glycosylated, N-acetyl-glucosaminated, prenylated, palmitoylated, and gamma-carboxylated amino acids or peptides.

31. The method according to claim 19 wherein said protein is myelin basic protein.

32. The method according to claim 31 wherein said protein is brain myelin basic protein.

33. The method according to claim 31 wherein said metabolic derivative is myelin basic protein-like material.

34. The method according to claim 31 wherein said accessible biological sample is urine.

35. The method according to claim 18, wherein said one or more inaccessible biological molecules is a lipid.

36. The method according to claim 35 wherein said lipid is a brain membrane lipid.

37. The method of claim 35 wherein said metabolic derivative is selected from the group consisting of 24(s)-hydroxycholesterol, galactosyl-cerebroside, sphingomyelin, and sphingosines.

38. The method according to claim 18 wherein said one or more inaccessible biological molecules is a polynucleotide.

39. The method according to claim 38 wherein said polynucleotide is deoxyribonucleic acid (DNA).

40. The method according to claim 39 wherein said label is introduced post-replication to said DNA.

41. The method according to claim 38 wherein said one or more metabolic derivatives is a nucleic acid with one or more nucleic acid residues.

42. The method according to claim 1 wherein said precursor molecule is administered repeatedly or continuously over a defined period of time before said step (b).

43. The method according to claim 38 wherein said metabolic derivative is selected from the group consisting of methyl-cytosine, a methylated base, 8-oxo-guanosine, an oxidatively modified base, deoxyribose, and ribose.

44. The method of claim 18, wherein said one or more inaccessible biological molecules is selected from glycosaminoglycans or proteoglycans.

45. The method of claim 44, wherein said one or more metabolic derivatives is selected from the group consisting of hyaluronic acid disaccharide, hyaluronic acid polymers, N-acetyl glucosamine, N-acetyl-galactosamine, chondroitin-sulfate disaccharide, chondroitin-sulfate polymers, heparin sulfate disaccharide, and heparin sulfate disaccharide polymers.

46. A method of identifying a disease state comprising assessing said biosynthesis or breakdown rate according to the method of claim 1, wherein said rate is indicative of a disease state.

47. The method according to claim 46 wherein said disease state is a physiological condition characterized by an alteration in said biosynthesis or breakdown rate of said one or more inaccessible biological molecules.

48. The method according to claim 46 wherein said disease state or condition is selected from the group consisting of osteoporosis, left-ventricular hypertrophy, liver cirrhosis, liver fibrosis, congestive heart failure, scleroderma, black-lung (coal-miner's pneumoconiosis), cardiac fibrosis, lung fibrosis, Alzheimer's disease, multiple sclerosis, rheumatoid arthritis, diabetes mellitus, muscle wasting syndromes, muscular dystrophies, athletic training, and cancer.



49. A method for monitoring a response of a disease state or a condition in an individual to a therapeutic intervention comprising the steps of:

- a) assessing said rate of biosynthesis or breakdown according to the method of claim 1, before the initiation of said therapeutic intervention;
- b) assessing the rate of biosynthesis or breakdown of said one or more inaccessible biological molecules according to the method of claim 1, after the initiation of such therapeutic intervention; and
- c) comparing the rates of biosynthesis or breakdown before and after therapeutic intervention to monitor the response of a disease or a condition to therapeutic intervention.

50. The method according to claim 49 wherein said disease state or condition is selected from the group consisting of osteoporosis, left-ventricular hypertrophy, liver cirrhosis, liver fibrosis, congestive heart failure, scleroderma, black-lung (coal-miner's pneumoconiosis), cardiac fibrosis, lung fibrosis, Alzheimer's disease, multiple sclerosis, rheumatoid arthritis, diabetes mellitus, muscle wasting syndromes, muscular dystrophies, athletic training, and cancer.

51. A method for monitoring a response of a disease state or a condition in an individual to a therapeutic intervention comprising the steps of:

- a) assessing said rate of biosynthesis or breakdown according to the method of claim 1; and
- b) comparing said rate of biosynthesis or breakdown to a reference rate of biosynthesis or breakdown of said one or more inaccessible biological molecules, wherein said reference rate is indicative of the disease state or of risk for the disease state.

52. The method according to claim 51 wherein said disease state or condition is selected from the group consisting of osteoporosis, left-ventricular hypertrophy, liver cirrhosis, liver fibrosis, congestive heart failure, scleroderma, black-lung (coal-miner's pneumoconiosis), cardiac fibrosis, lung fibrosis, Alzheimer's disease, multiple sclerosis, rheumatoid arthritis, diabetes mellitus, muscle wasting syndromes, muscular dystrophies, athletic training, and cancer.

53. A method for determining a whole-body pool size of said one or more inaccessible biological molecules in an individual comprising of the steps of:

- a) measuring the rate of biosynthesis of said inaccessible biological molecule by the method of claim 1;
- b) measuring the biosynthesis rate of said one or more inaccessible biological molecules; and
- c) dividing said daily excretion rate by the daily fractional replacement rate of said one or more metabolic derivatives to calculate whole-body pool size of said one or more inaccessible biological molecules in said individual.

54. A kit for determining the biosynthetic rate or breakdown rate of one or more inaccessible biological molecules in an individual comprising:

- a) an isotope-labeled precursor, and
- b) instructions for use of the kit,

wherein the kit is used to determine the biosynthetic rate or breakdown rate of said one or more inaccessible biological molecules in said individual.

55. The kit of claim 54 further comprising chemical compounds for isolating said one or more metabolic derivatives from urine, bone, or muscle.

56. The kit of claim 54 further comprising a tool for administration of precursor molecules.

57. The kit of claim 54 further comprising an instrument for collecting a sample from the subject.

58. A method of measuring the rate of biosynthesis or breakdown of a biological molecule comprising:

administering an isotope-labeled precursor molecule to an individual;  
obtaining an accessible biological sample comprising one or more metabolic derivatives of said inaccessible biological molecule from said individual;  
measuring the proportion of labeled to unlabeled metabolic derivatives at one or more times;

calculating the isotope enrichment of said inaccessible biological molecule by mass isotopomer distribution analysis (MIDA); and

applying precursor-product or exponential decay equations to determine said rate of biosynthesis or breakdown of said inaccessible biological molecule.

59. A method for determining the rate of biosynthesis or breakdown of collagen in an individual comprising the steps of:

a) administering deuterated water to an individual for a period of time sufficient for said deuterium to become incorporated into said collagen;

b) obtaining one or more accessible biological samples from an individual, wherein said one or more accessible biological samples comprise one or more collagen derivatives selecting from the group consisting of pyridinoline, deoxypyridinoline, hydroxyproline, hydroxylysine, glucosylgalactosyl-hydroxylysine, galactosylhydroxylysine, N-terminal telopeptide  $\alpha$ (I) (SEQ ID NO:1), N-terminal telopeptide  $\alpha$ 2(I) (SEQ ID NO:2), N-terminal telopeptide  $\alpha$ 2(I) (SEQ ID NO:3), N-terminal telopeptide  $\alpha$ 1(II) (SEQ ID NO:4), N-terminal telopeptide  $\alpha$ 1(III)

(SEQ ID NO:5), C-terminal telopeptide  $\alpha 1$ (I) (SEQ ID NO:6), C-terminal telopeptide  $\alpha 2$ (I) (SEQ ID NO:7), C-terminal telopeptide  $\alpha 1$ (II) (SEQ ID NO:8), C-terminal telopeptide  $\alpha 1$ (II) (SEQ ID NO:9), C-terminal telopeptide  $\alpha 1$ (II) (SEQ ID NO:10), C-terminal telopeptide  $\alpha 1$ (III) (SEQ ID NO:11), cross-linked carboxy-terminal peptide of type I collagen (ICTP), PINP( $\alpha 1$ ) (SEQ ID NO:12), PICP( $\alpha 1$ ) (SEQ ID NO:13), PINP( $\alpha 2$ ) (SEQ ID NO:14), PICP( $\alpha 2$ ) (SEQ ID NO:15), PIINP( $\alpha 1$ ) (SEQ ID NO:16), PIICP( $\alpha 1$ ) (SEQ ID NO:17), PIINP( $\alpha 1$ ) (SEQ ID NO:18), PIICP( $\alpha 1$ ) (SEQ ID NO:19), PIVNP( $\alpha 1$ ) (SEQ ID NO:20), PIVNP( $\alpha 2$ ) (SEQ ID NO:21), PIVNP( $\alpha 2$ ) (SEQ ID NO:22), PIVNP( $\alpha 3$ ) (SEQ ID NO:23), PIVNP( $\alpha 4$ ) (SEQ ID NO:24), PIVNP( $\alpha 5$ ) (SEQ ID NO:25), and PIVNP( $\alpha 6$ ) (SEQ ID NO:26);

- c) detecting said one or more collagen derivatives by mass spectrometry;
- d) calculating the isotope enrichment of said one or more collagen derivatives by mass isotopomer distribution analysis (MIDA); and
- e) applying precursor-product or exponential decay equations to determine the rate of biosynthesis or breakdown of said collagen.

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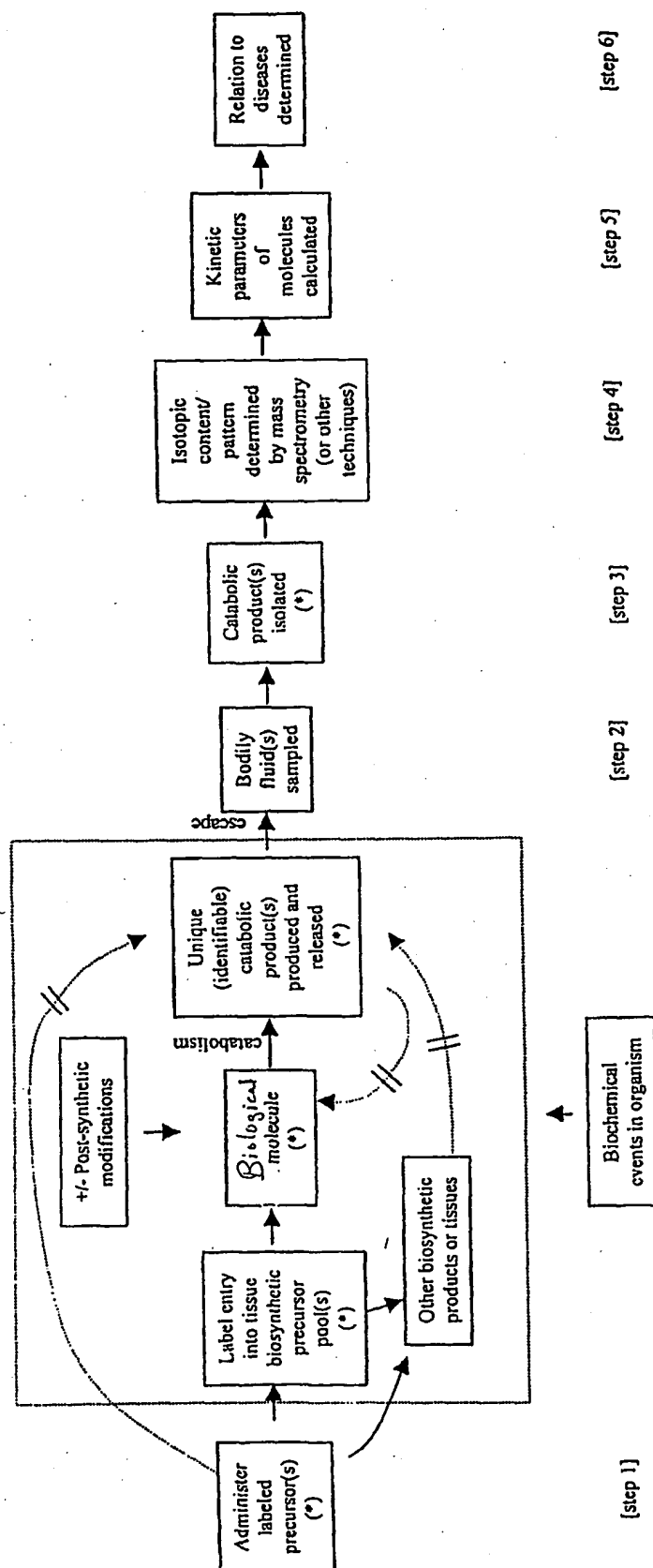


FIGURE 1

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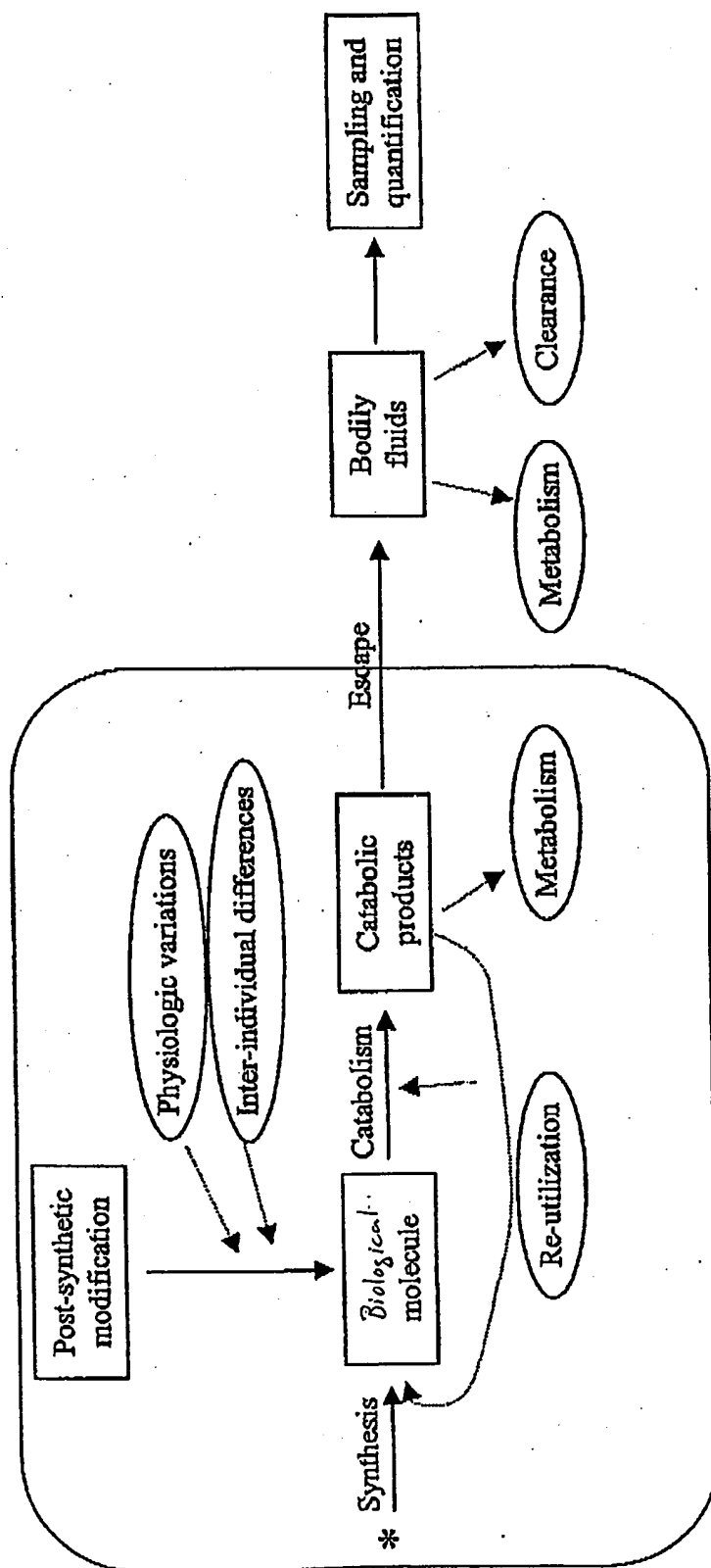


Figure 2

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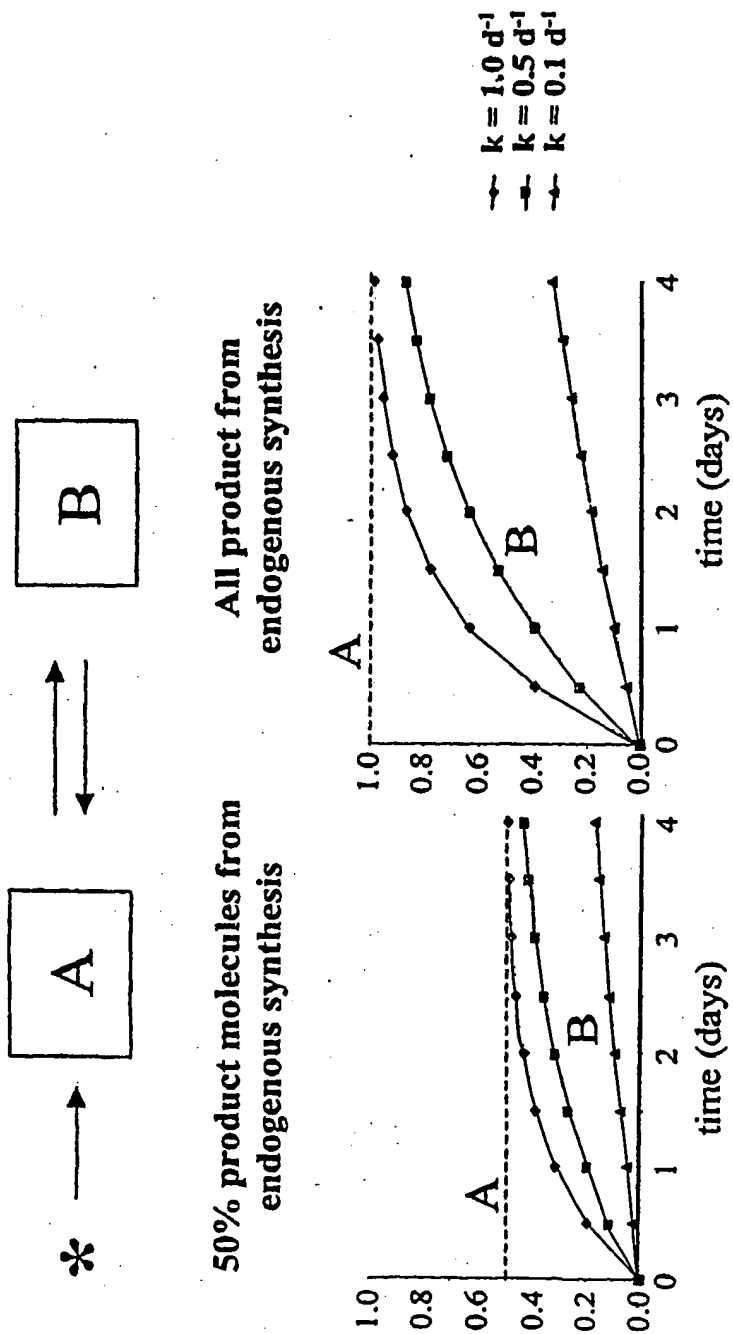


FIGURE 3

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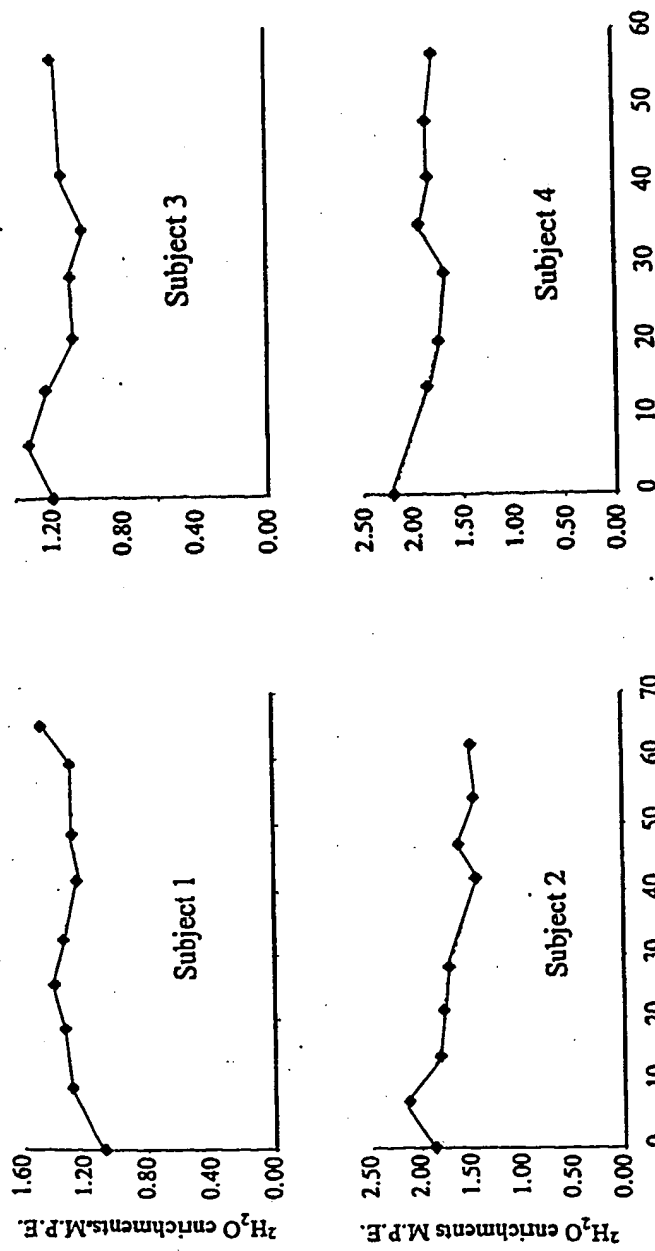


FIGURE 4



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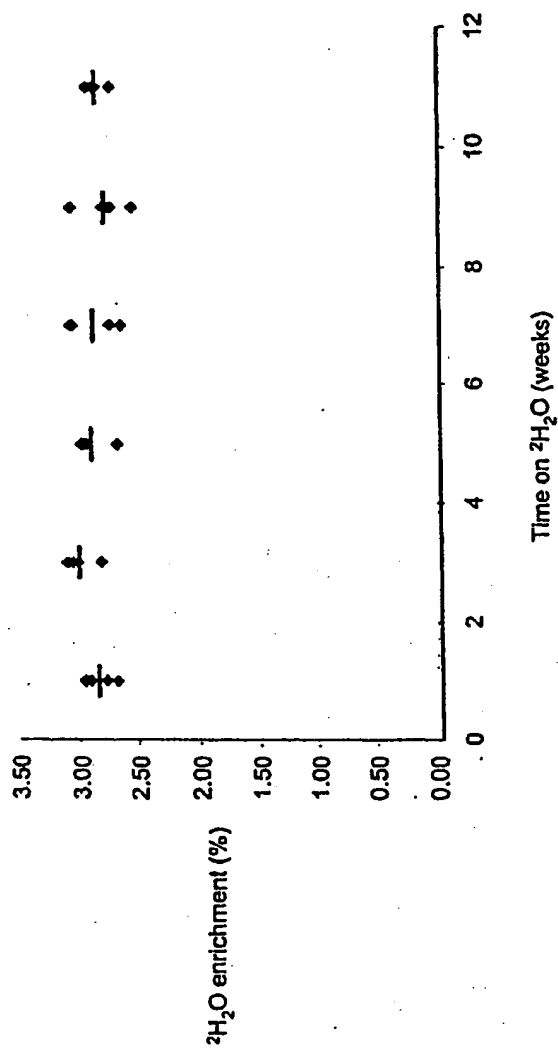


FIGURE 5

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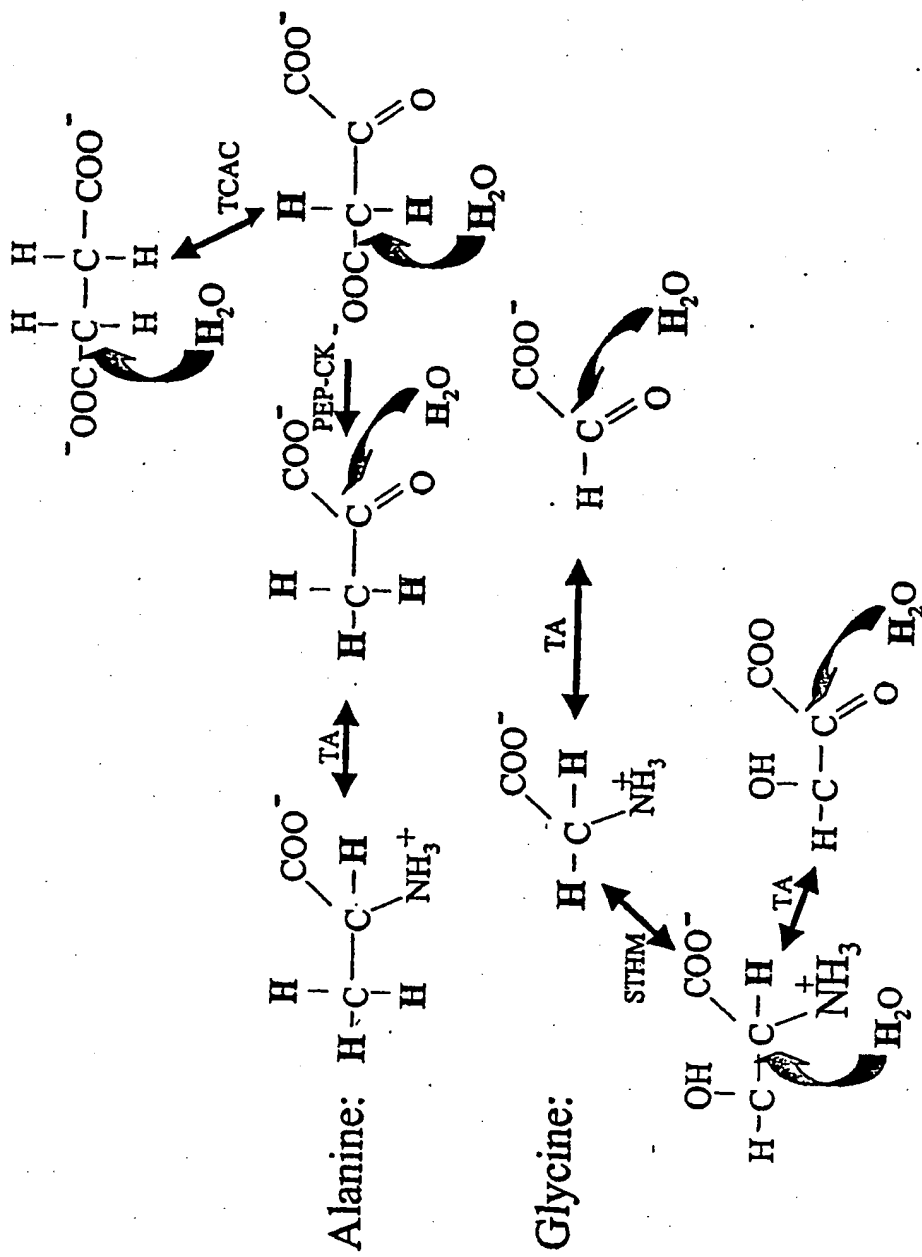


FIGURE 6A

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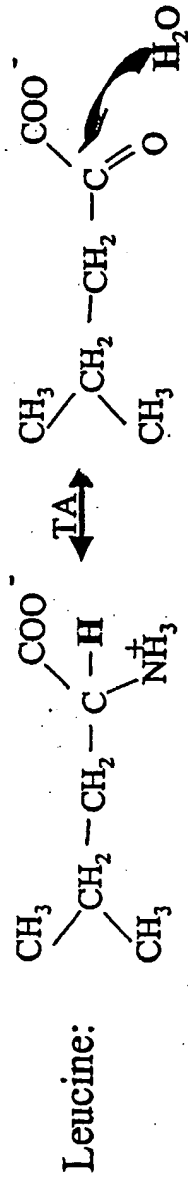


FIGURE 6B

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# $H_2^{18}O$ Labeling of Free Amino Acid for Protein Synthesis

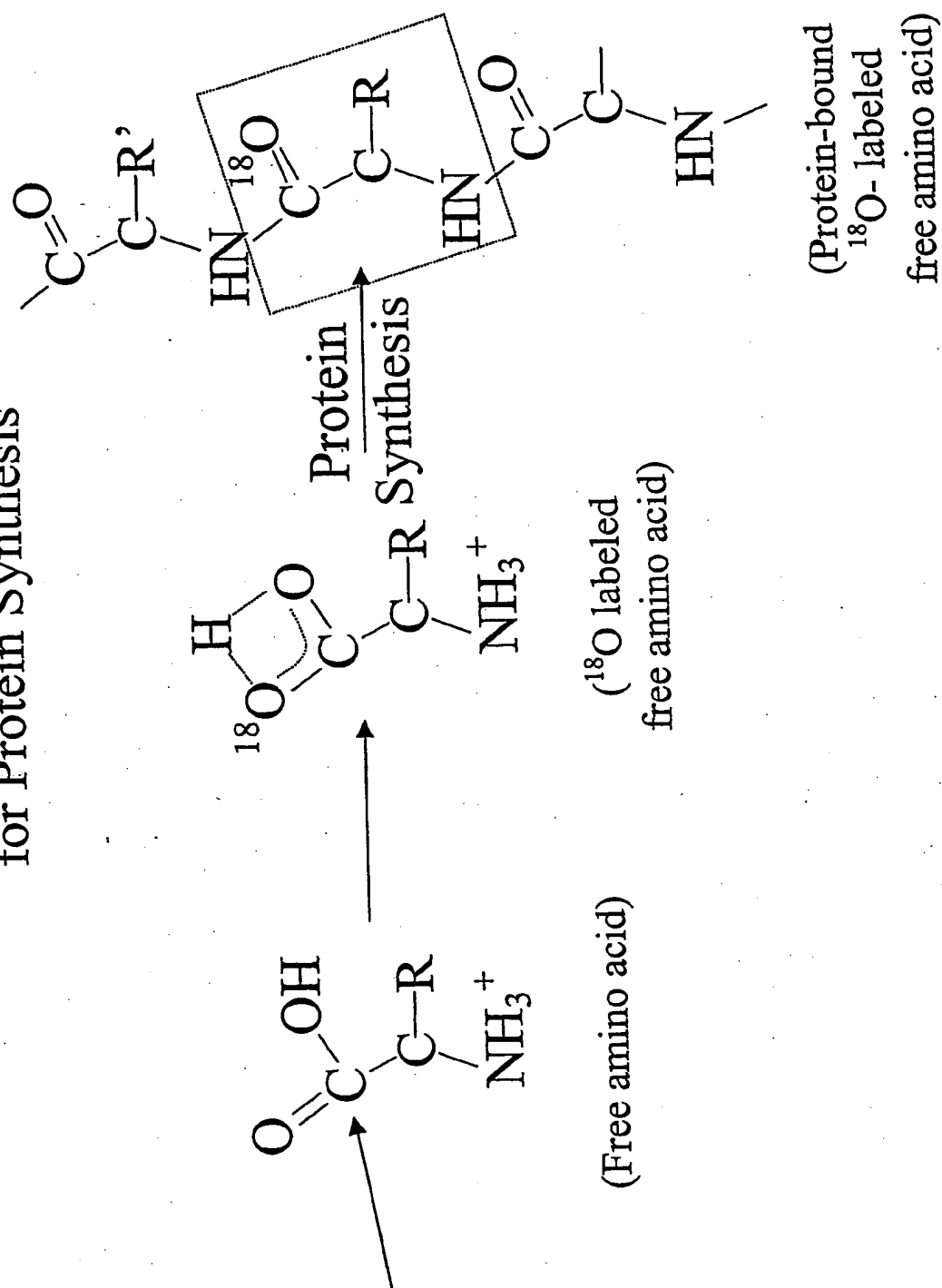


FIGURE 6C

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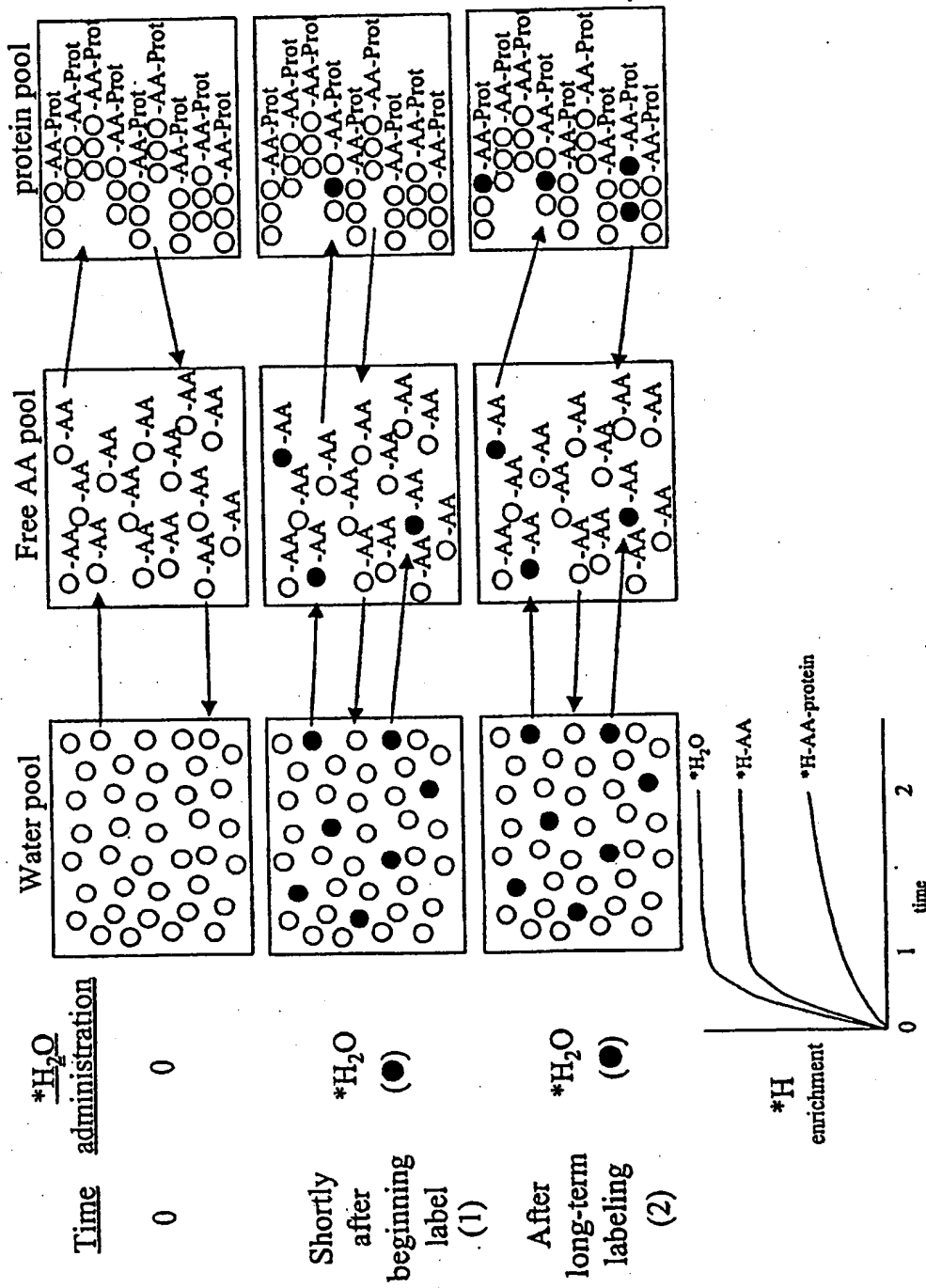


FIGURE 7

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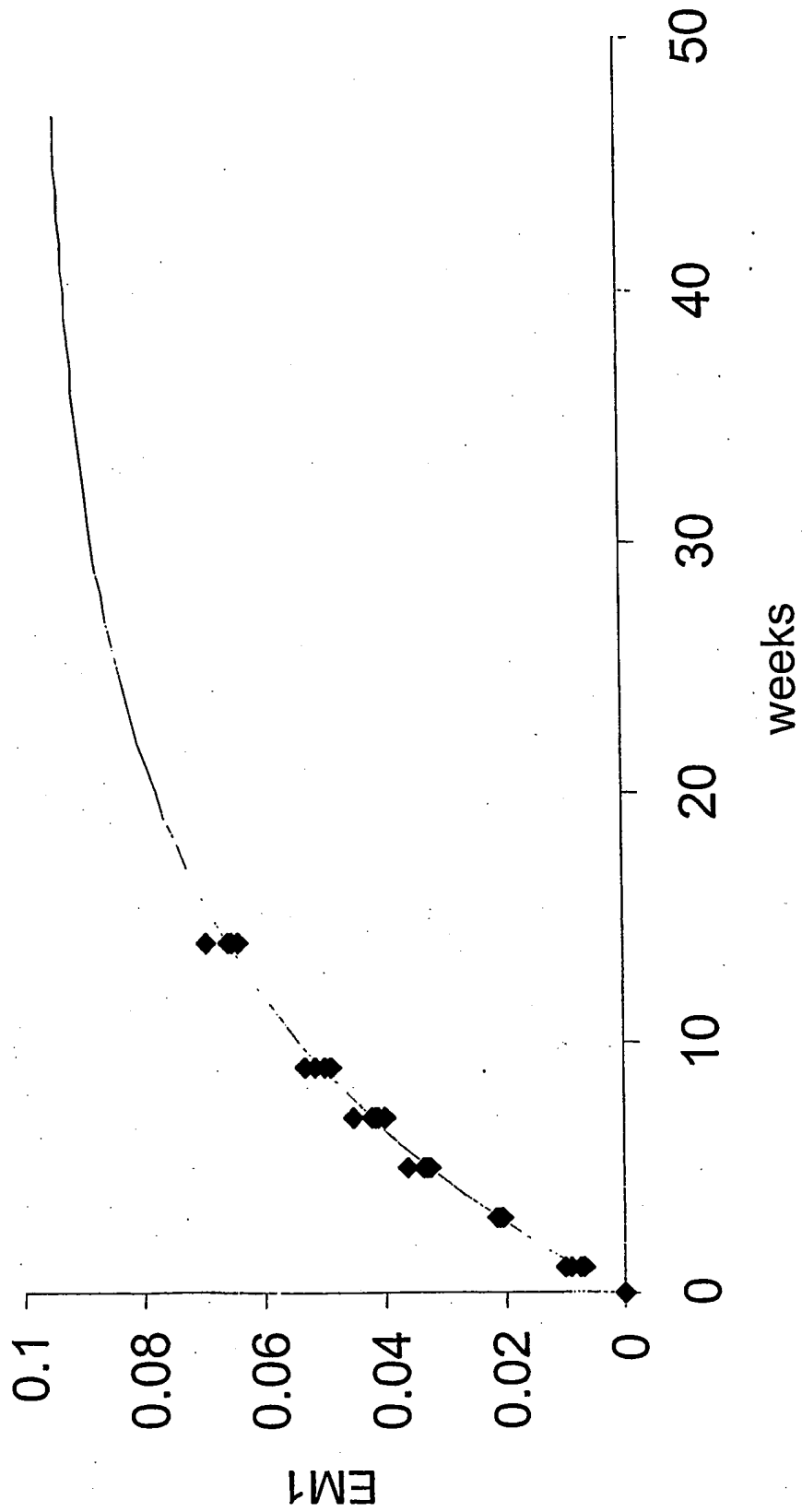


FIGURE 8A

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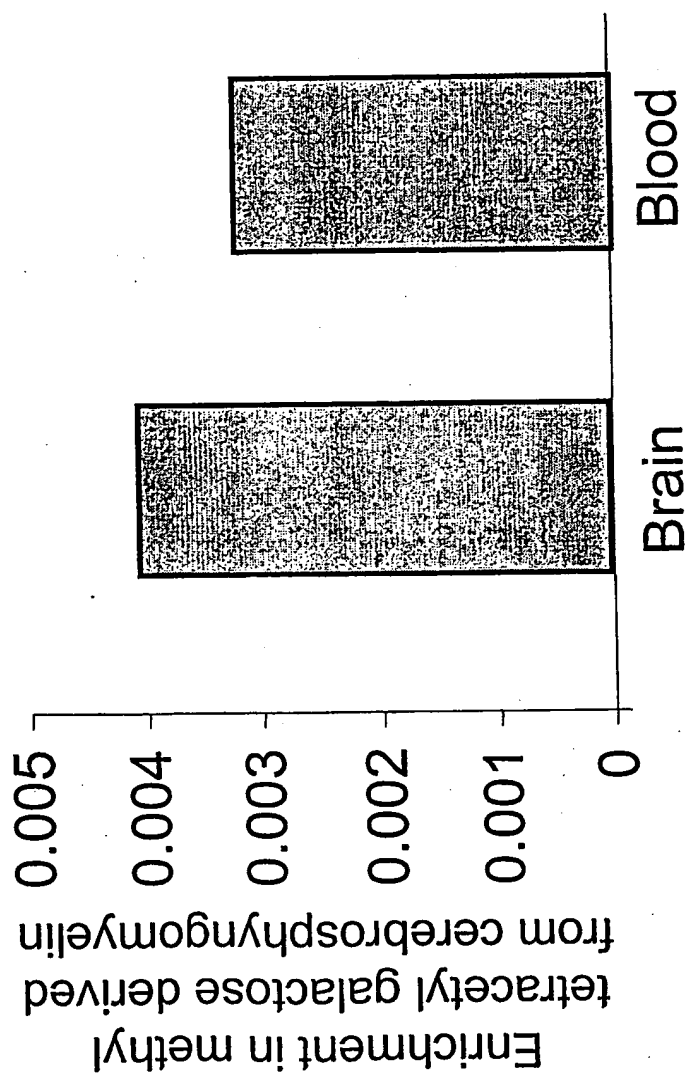


FIGURE 8B

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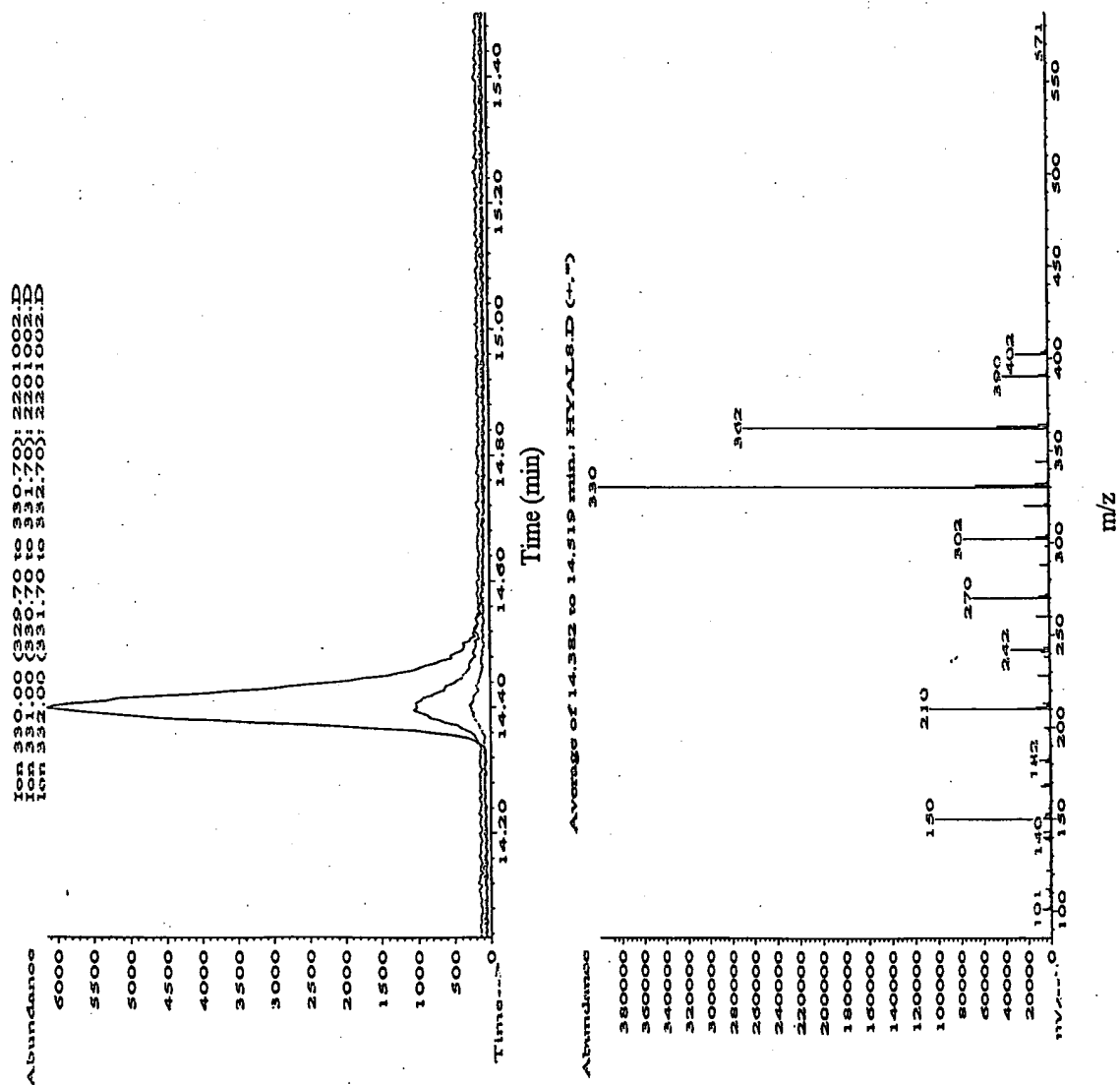


FIGURE 9



## SEQUENCE LISTING

<110> THE REGENTS OF THE UNIVERSITY OF CALIFORNIA

<120> MEASUREMENT OF BIOSYNTHESIS AND BREAKDOWN RATES OF  
BIOLOGICAL MOLECULES THAT ARE INACCESSIBLE OR NOT  
EASILY ACCESSIBLE TO DIRECT SAMPLING, NON-INVASIVELY,  
BY LABEL INCORPORATION INTO METABOLIC DERIVATIVES AND  
CATABOLITIC PRODUCTS

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<150> US 60/356,008

<151> 2002-02-12

<160> 28

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<213> Homo sapiens

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<210> 4

<211> 19

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<213> Homo sapiens

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Val Met Gln

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<210> 6  
<211> 26  
<212> PRT  
<213> Homo sapiens

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Gly Val Lys  
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 Asp Pro Leu Gln Tyr Met Arg Ala  
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 20 25

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 Pro Glu Pro Cys Arg Ile Cys Val Cys Asp Asn Gly Lys Val Leu Cys  
 35 40 45  
 Asp Asp Val Ile Cys Asp Glu Thr Lys Asn Cys Pro Gly Ala Glu Val  
 50 55 60  
 Pro Glu Gly Glu Cys Cys Pro Val Cys Pro Asp Gly Ser Glu Ser Pro  
 65 70 75 80  
 Thr Asp Gln Glu Thr Thr Gly Val Glu Gly Pro Lys Gly Asp Thr Gly  
 85 90 95  
 Pro Arg Gly Pro Arg Gly Pro Ala Gly Pro Pro Gly Arg Asp Gly Ile  
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 <213> Homo sapiens

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 Leu Lys Ser Leu Ser Gln Gln Ile Glu Asn Ile Arg Ser Pro Glu Gly  
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 Ser Arg Lys Asn Pro Ala Arg Thr Cys Arg Asp Leu Lys Met Cys His  
 35 40 45  
 Ser Asp Trp Lys Ser Gly Glu Tyr Trp Ile Asp Pro Asn Gln Gly Cys  
 50 55 60  
 Asn Leu Asp Ala Ile Lys Val Phe Cys Asn Met Glu Thr Gly Glu Thr  
 65 70 75 80  
 Cys Val Tyr Pro Thr Gln Pro Ser Val Ala Gln Lys Asn Trp Tyr Ile

				85					90					95			
Ser	Lys	Asn	Pro	Lys	Asp	Lys	Arg	His	Val	Trp	Phe	Gly	Glu	Ser	Met		
			100					105					110				
Thr	Asp	Gly	Phe	Gln	Phe	Glu	Tyr	Gly	Gly	Gln	Gly	Ser	Asp	Pro	Ala		
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Asp	Val	Ala	Ile	Gln	Leu	Thr	Phe	Leu	Arg	Leu	Met	Ser	Thr	Glu	Ala		
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Ser	Gln	Asn	Ile	Thr	Tyr	His	Cys	Lys	Asn	Ser	Val	Ala	Tyr	Met	Asp		
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Gln	Gln	Thr	Gly	Asn	Leu	Lys	Lys	Ala	Leu	Leu	Leu	Lys	Gly	Ser	Asn		
			165						170					175			
Glu	Ile	Glu	Ile	Arg	Ala	Glu	Gly	Asn	Ser	Arg	Phe	Thr	Tyr	Ser	Val		
			180					185					190				
Thr	Val	Asp	Gly	Cys	Thr	Ser	His	Thr	Gly	Ala	Trp	Gly	Lys	Thr	Val		
		195					200					205					
Ile	Glu	Tyr	Lys	Thr	Thr	Lys	Thr	Ser	Arg	Leu	Pro	Ile	Ile	Asp	Val		
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Ala	Pro	Leu	Asp	Val	Gly	Ala	Pro	Asp	Gln	Glu	Phe	Gly	Phe	Asp	Val		
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Gly	Pro	Val	Cys	Phe	Leu												
				245													

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 Gly Pro Thr Gly Pro Pro Gly Pro Pro Gly Pro Pro Gly Pro Pro Gly  
 35 40 45  
 Leu Gly Gly Asn Phe Ala Ala  
 50 55

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 <211> 265  
 <212> PRT  
 <213> Homo sapiens

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 Arg Ala Asp Gln Pro Arg Ser Ala Pro Ser Leu Arg Pro Lys Asp Tyr  
 20 25 30  
 Glu Val Asp Ala Thr Leu Lys Ser Leu Asn Asn Gln Ile Glu Thr Leu  
 35 40 45  
 Leu Thr Pro Glu Gly Ser Arg Lys Asn Pro Ala Arg Thr Cys Arg Asp  
 50 55 60  
 Leu Arg Leu Ser His Pro Glu Trp Ser Ser Gly Tyr Tyr Trp Ile Asp  
 65 70 75 80  
 Pro Asn Gln Gly Cys Thr Met Glu Ala Ile Lys Val Tyr Cys Asp Phe  
 85 90 95  
 Pro Thr Gly Glu Thr Cys Ile Arg Ala Gln Pro Glu Asn Ile Pro Ala  
 100 105 110  
 Lys Asn Trp Tyr Arg Ser Ser Lys Asp Lys Lys His Val Trp Leu Gly  
 115 120 125

Glu Thr Ile Asn Ala Gly Ser Gln Phe Glu Tyr Asn Val Glu Gly Val  
 130 135 140  
 Thr Ser Lys Glu Met Ala Thr Gln Leu Ala Phe Met Arg Leu Leu Ala  
 145 150 155 160  
 Asn Tyr Ala Ser Gln Asn Ile Thr Tyr His Cys Lys Asn Ser Ile Ala  
 165 170 175  
 Tyr Met Asp Glu Glu Thr Gly Asn Leu Lys Lys Ala Val Ile Leu Gln  
 180 185 190  
 Gly Ser Asn Asp Val Glu Leu Val Ala Glu Gly Asn Ser Arg Phe Thr  
 195 200 205  
 Tyr Thr Val Leu Val Asp Gly Cys Ser Lys Lys Thr Asn Glu Trp Gly  
 210 215 220  
 Lys Thr Ile Ile Glu Tyr Lys Thr Asn Lys Pro Ser Arg Leu Pro Phe  
 225 230 235 240  
 Leu Asp Ile Ala Pro Leu Asp Ile Gly Gly Ala Asp His Glu Phe Phe  
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 Val Asp Ile Gly Pro Val Cys Phe Lys  
 260 265

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 <213> Homo sapiens

<400> 16

Met Ile Arg Leu Gly Ala Pro Gln Ser Leu Val Leu Leu Thr Leu Leu  
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 20 25 30  
 Pro Lys Gly Gln Lys Gly Glu Pro Gly Asp Ile Lys Asp Ile Val Gly  
 35 40 45  
 Pro Lys Gly Pro Pro Gly Pro Gln Gly Pro Ala Gly Glu Gln Gly Pro  
 50 55 60  
 Arg Gly Asp Arg Gly Asp Lys Gly Glu Lys Gly Ala Pro Gly Pro Arg  
 65 70 75 80  
 Gly Arg Asp Gly Glu Pro Gly Thr Pro Gly Asn Pro Gly Pro Pro Gly  
 85 90 95  
 Pro Pro Gly Pro Pro Gly Pro Pro Gly Leu Gly Gly Asn Phe Ala Ala  
 100 105 110  
 Gln Met Ala Gly Gly Phe Asp Glu Lys Ala Gly Gly Ala Gln Leu Gly  
 115 120 125  
 Val Met Gln Gly Pro Met Gly Pro Met Gly Pro Arg Gly Pro Pro Gly  
 130 135 140  
 Pro Ala Gly Ala Pro Gly Pro Gln Gly Phe Gln Gly Asn Pro Gly Glu  
 145 150 155 160  
 Pro Gly Glu Pro Gly Val Ser  
 165

<210> 17  
 <211> 246  
 <212> PRT  
 <213> Homo sapiens

<400> 17

Asp Glu Ala Ala Gly Gly Leu Arg Gln His Asp Val Glu Val Asp Ala  
 1 5 10 15  
 Thr Leu Lys Ser Leu Asn Asn Gln Ile Glu Ser Ile Arg Ser Pro Glu  
 20 25 30  
 Gly Ser Lys Lys Asn Pro Ala Arg Thr Cys Arg Asp Ile Lys Leu Cys

35	40	45
His Pro Glu Trp Lys Ser Gly Asp Tyr Trp Ile Asp Pro Asn Gln Gly		
50	55	60
Cys Thr Leu Asp Ala Ile Lys Val Phe Cys Asn Met Glu Thr Gly Glu		
65	70	75
Thr Cys Val Tyr Pro Thr Pro Ser Ser Ile Pro Arg Lys Asn Trp Trp		
85	90	95
Thr Ser Lys Thr Lys Asp Lys Lys His Val Trp Phe Ala Glu Thr Ile		
100	105	110
Asn Gly Gly Phe His Phe Ser Tyr Gly Asp Glu Asn Leu Ser Pro Asn		
115	120	125
Thr Ala Ser Ile Gln Met Thr Phe Leu Arg Leu Leu Ser Thr Glu Gly		
130	135	140
Ser Gln Asn Val Thr Tyr His Cys Lys Asn Ser Ile Ala Tyr Met Asp		
145	150	155
Glu Glu Thr Gly Asn Leu Lys Lys Ala Ile Leu Ile Gln Gly Ser Asn		
165	170	175
Asp Val Glu Ile Arg Ala Glu Gly Asn Ser Arg Phe Thr Tyr Ser Val		
180	185	190
Leu Glu Asp Gly Cys Thr Lys His Thr Gly Lys Trp Gly Lys Thr Val		
195	200	205
Ile Glu Tyr Arg Ser Gln Lys Thr Ser Arg Leu Pro Ile Val Asp Ile		
210	215	220
Ala Pro Met Asp Ile Gly Gly Ala Asp Gln Glu Phe Gly Val Asp Ile		
225	230	235
Gly Pro Val Cys Phe Leu		240
245		

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&lt;211&gt; 125

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 18

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Ala Asp Arg Asp Val Trp Lys Pro Glu Pro Cys Gln Ile Cys Val Cys	10
20	25
Asp Ser Gly Ser Val Leu Cys Asp Asp Ile Ile Cys Asp Asp Gln Glu	30
35	40
Leu Asp Cys Pro Asn Pro Glu Ile Pro Phe Gly Glu Cys Cys Ala Val	45
50	55
Cys Pro Gln Pro Pro Thr Ala Pro Thr Arg Pro Pro Asn Gly Gln Gly	60
65	70
Pro Gln Gly Pro Lys Gly Asp Pro Gly Pro Pro Gly Ile Pro Gly Arg	75
85	90
Asn Gly Asp Pro Gly Ile Pro Gly Gln Pro Gly Ser Pro Gly Ser Pro	95
100	105
Gly Pro Pro Gly Ile Cys Glu Ser Cys Pro Thr Gly Pro	110
115	120
	125

&lt;210&gt; 19

&lt;211&gt; 262

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 19

Ile Ala Gly Ile Gly Gly Glu Lys Ala Gly Gly Phe Ala Pro Tyr Tyr	
1	5
	10
	15

Gly Asp Glu Pro Met Asp Phe Lys Ile Asn Thr Asp Glu Ile Met Thr  
                   20                                  25                                  30  
 Ser Leu Lys Ser Val Asn Gly Gln Ile Glu Ser Leu Ile Ser Pro Asp  
                   35                                  40                                  45  
 Gly Ser Arg Lys Asn Pro Ala Arg Asn Cys Arg Asp Leu Lys Phe Cys  
                   50                                  55                                  60  
 His Pro Glu Leu Lys Ser Gly Glu Tyr Trp Val Asp Pro Asn Gln Gly  
 65                                  70                                  75                                  80  
 Cys Lys Leu Asp Ala Ile Lys Val Phe Cys Asn Met Glu Thr Gly Glu  
                                   85                                  90                                  95  
 Thr Cys Ile Ser Ala Asn Pro Leu Asn Val Pro Arg Lys His Trp Trp  
                                   100                                  105                                  110  
 Thr Asp Ser Ser Ala Glu Lys Lys His Val Trp Phe Gly Glu Ser Met  
                                   115                                  120                                  125  
 Asp Gly Gly Phe Gln Phe Ser Tyr Gly Asn Pro Glu Leu Pro Glu Asp  
                   130                                  135                                  140  
 Val Leu Asp Val Gln Leu Ala Phe Leu Arg Leu Leu Ser Ser Arg Ala  
 145                                  150                                  155                                  160  
 Ser Gln Asn Ile Thr Tyr His Cys Lys Asn Ser Ile Ala Tyr Met Asp  
                                   165                                  170                                  175  
 Gln Ala Ser Gly Asn Val Lys Lys Ala Leu Lys Leu Met Gly Ser Asn  
                                   180                                  185                                  190  
 Glu Gly Glu Phe Lys Ala Glu Gly Asn Ser Lys Phe Thr Tyr Thr Val  
                                   195                                  200                                  205  
 Leu Glu Asp Gly Cys Thr Lys His Thr Gly Glu Trp Ser Lys Thr Val  
                                   210                                  215                                  220  
 Phe Glu Tyr Arg Thr Arg Lys Ala Val Arg Leu Pro Ile Val Asp Ile  
 225                                  230                                  235                                  240  
 Ala Pro Tyr Asp Ile Gly Gly Pro Asp Gln Glu Phe Gly Val Asp Val  
                                   245                                  250                                  255  
 Gly Pro Val Cys Phe Leu  
                                   260

&lt;210&gt; 20

&lt;211&gt; 26

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 20

Met Gly Pro Arg Leu Ser Val Trp Leu Leu Leu Leu Pro Ala Ala Leu  
   1                                  5                                  10                                  15  
 Leu Leu His Glu Glu His Ser Arg Ala Ala  
                                   20                                  25

&lt;210&gt; 21

&lt;211&gt; 28

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 21

Met Gly Arg Asp Gln Arg Ala Val Ala Gly Pro Ala Leu Arg Arg Trp  
   1                                  5                                  10                                  15  
 Leu Leu Leu Gly Thr Val Thr Val Gly Phe Leu Ala  
                                   20                                  25

&lt;210&gt; 22

&lt;211&gt; 158

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 22

Leu Leu Ala Gln Ser Val Leu Gly Gly Val Lys Lys Leu Asp Val Pro  
 1 5 10 15  
 Cys Gly Gly Arg Asp Cys Ser Gly Gly Cys Gln Cys Tyr Pro Glu Lys  
 20 25 30  
 Gly Ala Arg Gly Gln Pro Gly Ala Val Gly Pro Gln Gly Tyr Asn Gly  
 35 40 45  
 Pro Pro Gly Leu Gln Gly Phe Pro Gly Leu Gln Gly Arg Lys Gly Asp  
 50 55 60  
 Lys Gly Glu Arg Gly Val Pro Gly Pro Thr Gly Pro Lys Gly Asp Val  
 65 70 75 80  
 Gly Ala Arg Gly Val Ser Gly Phe Pro Gly Ala Asp Gly Ile Pro Gly  
 85 90 95  
 His Pro Gly Gln Gly Gly Pro Arg Gly Arg Pro Gly Tyr Asp Gly Cys  
 100 105 110  
 Asn Gly Thr Arg Gly Asp Ala Gly Pro Gln Gly Pro Ser Gly Ser Gly  
 115 120 125  
 Gly Phe Pro Gly Leu Pro Gly Pro Gln Gly Pro Lys Gly Gln Lys Gly  
 130 135 140  
 Glu Pro Tyr Ala Leu Ser Lys Glu Asp Arg Asp Lys Tyr Arg  
 145 150 155

&lt;210&gt; 23

&lt;211&gt; 28

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 23

Met Ser Ala Arg Thr Ala Pro Arg Pro Gln Val Leu Leu Leu Pro Leu  
 1 5 10 15  
 Leu Leu Val Leu Leu Ala Ala Ala Pro Ala Ala Ser  
 20 25

&lt;210&gt; 24

&lt;211&gt; 38

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 24

Met Trp Ser Leu His Ile Val Leu Met Arg Cys Ser Phe Arg Leu Thr  
 1 5 10 15  
 Lys Ser Leu Ala Thr Gly Pro Trp Ser Leu Ile Leu Ile Leu Phe Ser  
 20 25 30  
 Val Gln Tyr Val Tyr Gly  
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&lt;210&gt; 25

&lt;211&gt; 26

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 25

Met Lys Leu Arg Gly Val Ser Leu Ala Ala Gly Leu Phe Leu Leu Ala  
 1 5 10 15  
 Leu Ser Leu Trp Gly Gln Pro Ala Glu Ala  
 20 25



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 Glu Glu Leu Ala Ala  
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 20 25 30  
 Gly Leu Met Val Gly Gly Val Val  
 35 40

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 Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile  
 20 25 30  
 Gly Leu Met Val Gly Gly Val Val Ile Ala  
 35 40

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